ANTI-CTLA4 AND ANTI-PD-1 BIFUNCTIONAL ANTIBODY, PHARMACEUTICAL COMPOSITION THEREOF AND USE THEREOF

TECHNICAL FIELD

The present invention belongs to the field of tumor therapy and molecular immunology. The present invention relates to anti-CTLA4 and anti-PD-1 bispecific antibodies, and their pharmaceutical compositions and methods of use. Specifically, the present invention relates to monoclonal antibodies against CTLA4 and PD-1 proteins.

TECHNICAL BACKGROUND

Cytotoxic T lymphocyte associated antigen 4 (CTLA4) closely relates to CD28 in gene structure, chromosomal localization, homology of sequences and gene expression, and both of them are receptors of costimulatory molecule B7, and mainly expresses on the cell surface of activated T cells. Interaction of CTLA4 and B7 inhibits the activation of T cells in mice and human, and negatively regulates the activation of T cells.

Anti-CTLA4 antibody or CTLA4 ligand can prevent CTLA4 from binding to its natural ligand, thereby block the negative signal transduction in T cells induced by CTLA4, and enhance the response of T cells to various antigens, which has been confirmed by both in vivo and in vitro studies. Currently, clinical trials of anti-CTLA4 antibodies treating prostate cancer, bladder cancer, colorectal cancer, gastrointestinal cancer, liver cancer, and malignant melanoma, etc. are ongoing (Grosso JF., Jure-Kunkel MN., CTLA-4 blockade in tumor models: an overview of preclinical and translational research. Cancer Immun. 2013; 13:5. Epub 2013 Jan 22).

Interleukin 2 (IL-2) produced by T cells is a cytokine regulating proliferation of certain subgroups of T cells, and a crucial factor regulating immune responses, promoting the proliferation of activated B cells and participating in antibody responses, hematopoiesis and tumor surveillance. Recombinant human IL-2 has been approved by the U. S. FDA for the treatment of malignant tumors (including melanoma, renal tumors, etc.) while undergoing clinical studies to treat chronic viral infections (Chavez, A.R., et al., Pharmacologic administration of interleukin-2. Ann N Y Acad Sci, 2009. 1182: p.14-27). In in vitro experiments, anti-CTLA4 antibodies can specifically remove the

immunosuppression of CTLA4, activate T cells, and induce the generation of IL-2, displaying promising prospects in therapies for neoplastic and parasitic diseases.

As crucial factors on T cell functions, CTLA4 and anti-CTLA4 antibodies have particular therapeutic effects via intervening immune microenvironment, displaying high efficacy and supplementing traditional medicine, thereby crafting new opportunities in therapies. The therapeutic effects of CTLA4 and anti-CTLA4 antibodies are investigated in various pre-clinical and clinical studies, such as inhibition of airway hyper-responsiveness in asthma animal models, prevention of the development of rheumatic diseases, and induction of immune tolerance in allogeneic transplantation, etc. Meanwhile, although no adverse effects have been found in short-term clinical trials, we should note the potential impacts of long-term usage of drugs targeting CTLA4, such as anti-CTLA4 antibody, might provoke autoimmune diseases due to over-blockage on CTLA4-B7 signal pathway. Since antibodies can bind specifically to its antigen and induce target cell lysis or block pathological progress, drug development of antibodies especially humanized antibodies is very important in treating malignant tumors or auto-immune diseases.

The transmembrane receptor PD-1 (programmed cell death 1, also known as PD-1) is a member of the CD28 gene family, expresses in activated T cells, B cells and myeloid cells. Receptors of PD-1, PDL1 and PDL2, belong to the B7 superfamily; wherein PDL1 is broadly expressed in a variety of cells including T cells, B cells, endothelial cells and epithelial cells, while PDL2 is only expressed in antigen presenting cells such as dendritic cells and macrophages.

T cells play an important role in clearing viral infections, but T cell antivirus responses are often associated with immunopathogenesis. Although negative regulation of T cell activation mediated by PD-1 is critical in reducing tissue damage caused by infection, blocking or inhibiting the PD-1 pathway might lead to autoimmune diseases, for example, PD-1 gene knockout mice showed more effective clearance of pancreatic virus infection, but led to more severe liver damage (Iasi et al., 2003, j. Exp. J Med, 198, 39-50). In addition, tumors with high PD-1 expression often develop into cancers that are difficult to detect (Hamanishi et al., 2007, Proc. Natl. Acad. Sci. USA 104:3360-5). An

established method to regulate PD-1 expression is through injection of antibodies in vivo.

Due to the broad antitumor prospects and astounding efficacy of PD-1 antibodies, it is generally believed that antibodies against PD-1 pathways will lead to breakthroughs in the treatment of a variety of tumors: non-small cell lung cancer, renal cell carcinoma, ovarian cancer, melanoma (Homet M. B., Parisi G., et al., Anti-PD-1 Therapy in Melanoma. Semin Oncol. 2015 Jun;42(3):466-473), leukemia and anemia (Held SA, Heine A, et al., Advances in immunotherapy of chronic myeloid leukemia CML. Curr Cancer Drug Targets. 2013 Sep;13(7):768-74). Ever since the revelation of the unprecedented clinical efficacy data at the annual meetings of American Association for Cancer Research (AACR) and American Society of Clinical Oncology (ASCO) in 2012 and 2013, PD-1 antibodies have become the hottest new drugs in R&D in the global pharmaceutical industry.

Interferon gamma (IFN-γ) is produced naturally mainly by natural killer (NK) cells, natural killer T (NKT) cells, or by effector T cells consisting of $CD4^+$ Th1 cells and $CD8^+$ cytotoxic T lymphocytes (CTL) after being stimulated by specific antigens. As an important innate immune and acquired immune cytokine, IFN-γ plays an import role in antagonizing or inhibiting viral, some bacterial and protozoon infections. In the meantime, IFN-γ can activate macrophages and induce the expression of type 2 major histocompatibility complex (MHC) to activate immune responses to control the progression of tumors (SchoenbornJR, Wilson CB. Regulation of Interferon-gamma Durin g Innate and Adaptive Immune Responses. Advances in Immunology 2007; 96: 41-101) .

Monoclonal antibodies (mAbs) targeting a single antigen have been used to treat cancers, inflammation, infectious diseases, etc. However, the cause and in vivo factors of many diseases are complicated, including up- or down-regulations of different proteins, cytokines or receptors in different signaling pathways, either inhibiting or promoting biological functions. Therefore, simultaneously blocking different targets can improve treatment efficacy, which can be achieved by combinations of drugs with different targets or by one drug with multiple targets, such as multispecific antibodies.

Bispecific antibody, also called bi-functional antibody targeting two different

antigens at the same time, can be produced by immune sorting purification, as well as advantageous recombinant technologies with flexibilities in binding site optimization, format of synthesis, and production output. At present, there have been more than 45 forms of bispecific antibodies (Müller D, Kontermann RE. Bispecific antibodies for cancer immunotherapy: Current perspectives. BioDrugs 2010; 24:89-98). The IgG-scFv structure, named after Morrison, has been used in many bispecific antibodies, (1997 Coloma MJ, Morrison SL. The Design and production of will be tetravalent bispecific antibodies. Nat Biotechnol. Nature Biotechnology, 1997; 15, 15, 9-163). Bispecific antibody with IgG-scFv structure has been proven as an ideal form of bispecific antibody with advantages in antibody engineering, expression and purification due to its similarity to the natural IgG format (Miller BR, Demarest SJ, et al., Stability engineering of scFvs for the development of bispecific and multivalent antibodies. Protein Eng Des Sel 2010; 23:549-57; Fitzgerald J, Lugovskoy A. Rational engineering of antibody therapeutics targeting multiple oncogene pathways. MAbs 2011; 3:299-309).

However, bispecific antibodies have been developed mostly against antigens on the surface of two different cells, not against two different antigens on the same cell. Thus, a bispecific antibody drug against both PD-1 and CTLA4 needs to be developed.

SUMMARY

Through profound research and creative work, by immunizing mice with recombinant PD-1 or CTLA4 expressed in mammalian cells as antigen, the inventors obtained hybridoma cells via fusion of mouse splenocytes and myeloma cells. After screening a large number of samples, the inventors obtained the following hybridoma cell lines respectively:

Hybridoma cell line LT002 (CTLA4-4G10), which was preserved in China Center for Type Culture Collection (CCTCC) on June 16, 2015, with the CCTCC Deposit Accession NO: C201587;

and

hybridoma cell line LT003 (PD-1-14C12), which was preserved in China Center for Type Culture Collection (CCTCC) on June 16, 2015, with the CCTCC Deposit Accession

NO: C2015105.

The inventors surprisingly found that:

Hybridoma cell line LT002 is capable of secreting a specific monoclonal antibody (named 4G10) that binds specifically to CTLA4, and the monoclonal antibody can effectively block the interaction of CTLA4 to B7.

Hybridoma cell line LT003 is capable of secreting a specific monoclonal antibody (named 14C12) that specifically binds to PD-1, and the monoclonal antibody can effectively block the interaction of PD-1 to PDL1.

Furthermore, the inventors generated humanized antibodies against CTLA4 (named 4G10H1L1, 4G10H3L3, 4G10H4L3 and 8D2H14L2, respectively) and humanized antibodies against PD-1 (named 14C12H1L1) in a creative way.

Furthermore, the inventors created a series of new humanized bispecific antibodies (named BiAb001 BiAb002 BiAb003, BiAb004, BiAb007 and BiAb010, respectively) via recombining the two kinds of humanized antibodies, which can bind both CTLA4 and PD-1, and block interactions of CTLA4 with B7, and PD-1 with PDL1. The bispecific antibodies can effectively bind and activate human T cells, induce lymphocytes to secrete IFN-γ and IL-2, with the potential to be prepared into drugs for prevention and treatment of cancers, such as lung cancer, melanoma, renal cancer, ovarian cancer and leukemia.

The following are provided by the present disclosure:

In one example, there is provided a bispecific antibody thereof, wherein,

the first protein functional area targets PD-1,

and

the second protein functional area targets CTLA4.

In one aspect, the present disclosure refers to a bispecific antibody comprising at least one first protein functional area that binds to PD-1 and at least one second protein functional area that binds to CTLA4, wherein the at least one first and at least one second protein functional areas each comprise an immunoglobulin or antigen-binding fragment thereof,

wherein the at least one first protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 29-31 and a

light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 32-34,

wherein the at least one second protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35-37 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40.

In another aspect, the present disclosure refers to a bispecific antibody comprising at least one first protein functional area that binds to PD-1 and at least one second protein functional area that binds to CTLA4, wherein the at least one first and at least one second protein functional areas each comprise an immunoglobulin or antigen-binding fragment thereof,

wherein the at least one first protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 29-31 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 32-34,

wherein the at least one second protein functional area comprises comprising a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35, 41 and 37 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40.

In another aspect, the present disclosure refers to a bispecific antibody comprising at least one first protein functional area that binds to PD-1 and at least one second protein functional area that binds to CTLA4, wherein the at least one first and at least one second protein functional areas each comprise an immunoglobulin or antigen-binding fragment thereof,

wherein the at least one first protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 29-31 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 32-34,

wherein the at least one second protein functional area comprises a heavy chain

variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 42-44 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 45-47.

In one example of the disclosure, the said bispecific antibody, wherein the said first and second protein functional areas are connected directly or via connecting fragments; Preferably, the connecting fragments are (GGGGS)n, and n is a positive integer, such as 1, 2, 3, 4, 5 or 6.

In one example of the disclosure, the said bispecific antibody, wherein,

the said first and second protein functional areas are respectively immunoglobulins

or their antigen-binding fragments, such as half antibody, Fab, F(ab')2 or single-chain antibody.

Preferably, the said first protein functional area is an immunoglobulin, and the said second protein functional area is a single-chain antibody;

or,

Preferably, the said first protein functional area is a single-chain antibody, and the said second protein functional area is an immunoglobulin.

In one example of the disclosure, the said bispecific antibody, wherein, the quantity of the first protein functional area or the second protein functional area is one, two, or more, independently.

In one example of the disclosure, the said bispecific antibody, wherein, the said immunoglobulin is IgG, IgA, IgD, IgE or IgM; Preferably IgG, such as IgG1, IgG2, IgG3, or IgG4.

In one example of the disclosure, the said bispecific antibody, wherein, the said single-chain antibody is attached at the c-terminal of the heavy chain of the immunoglobulin. Since one immunoglobulin consists of two heavy chains, thus one immunoglobulin molecule is linked to two single-chain antibody molecules. Preferably, the two said single-chain antibody molecules are the same.

In one example of the disclosure, the said bispecific antibody, wherein,

the heavy chain variable region of the said immunoglobulin comprises CDRs with the amino acid sequences of SEQ ID NO: 29-31, and the light chain variable region of the said immunoglobulin comprises CDRs with the amino acid sequences of SEQ ID NO: 32-34;

And/or,

the heavy chain variable region of the said single-chain antibody comprises CDRs with the amino acid sequences of SEQ ID NO: 35 – 37, or SEQ ID NO: 35, SEQ ID NO: 41 and SEQ ID NO: 37, or SEQ ID NO: 42-44; and the light chain variable region of the said single-chain antibody comprises CDRs with the amino acid sequences of SEQ ID NO: 38-40, or SEQ ID NO: 45-47.

In one example of the disclosure, the said bispecific antibody, wherein,

the heavy chain variable region of the said immunoglobulin comprises CDRs with the amino acid sequences of SEQ ID NO: 35-37, or SEQ ID NO: 35, SEQ ID NO: 41 and SEQ ID NO: 37, or SEQ ID NO: 42-44; and the light chain variable region of the said immunoglobulin comprises CDRs with the amino acid sequences of SEQ ID NO: 38-40, or SEQ ID NO: 45-47;

and/or,

the heavy chain variable region of the said single-chain antibody comprises CDRs with the amino acid sequences of SEQ ID NO: 29-31; and the light chain variable region of the said single-chain antibody comprises CDRs with the amino acid sequences of SEQ ID NO: 32-34.

In one example of the disclosure, the said bispecific antibody, wherein,

the amino acid sequence of the heavy chain variable region of the said immunoglobulin is selected from SEQ ID NO. 16 or SEQ ID NO. 20; the amino acid sequence of the light chain variable region of the said immunoglobulin is selected from SEQ ID NO: 18 or SEQ ID NO: 22;

And/or,

the amino acid sequence of the heavy chain variable region of the said single-chain antibody is selected from SEQ ID NO: 2, SEQ ID NO: 6, SEQ ID NO: 10, SEQ ID NO: 14 or SEQ ID NO: 25; the amino acid sequence of the light chain variable region of the said single chain antibody is selected from SEQ ID NO: 4, SEQ ID NO: 8, SEQ ID NO: 12 or SEQ ID NO: 27.

In one example of the disclosure, the said bispecific antibody, wherein,

the amino acid sequence of the heavy chain variable region of the said immunoglobulin is selected from SEQ ID NO: 2, SEQ ID NO: 6, SEQ ID NO: 10, SEQ ID NO: 14 or SEQ ID NO: 25; the amino acid sequence of the light chain variable region of the single-chain antibody is selected from SEQ ID NO: 4, SEQ ID NO:8, SEQ ID NO:12 or SEQ ID NO:27;

and/or,

The amino acid sequence of the heavy chain variable region of the said single-chain antibody is selected from SEQ ID NO: 16 or SEQ ID NO: 20; the amino acid sequence of

the light chain variable region of the said immunoglobulin is selected from SEQ ID NO: 18 or SEQ ID NO: 22.

In one example of the disclosure, the said bispecific antibody, wherein,

the said immunoglobulin contains non-CDR regions from species other than mouse, for example, from human.

In one example of the disclosure, the constant region of the said immunoglobulin is humanized. For example, the constant region of the heavy chain is Ig gamma-1 chain C region, ACCESSION: P01857; the constant region of light chain is Ig kappa chain C region, ACCESSION: P01834.

In one example of the disclosure, the said bispecific antibody, wherein, the said bispecific antibody binds to CTLA4 protein and/or PD-1 protein with a K_D less than approximately 10^{-5} M, such as less than approximately 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M or 10^{-10} M or less.

The present disclosure also relates to a bispecific antibody, whose heavy chain variable region comprises CDRs with amino acid sequences of SEQ ID NO: 29-31, SEQ ID NO: 35-37, or SEQ ID NO: 35, SEQ ID NO: 41, and SEQ ID NO: 37, or SEQ ID NO: 42-44;

and,

of SEQ ID NO: 32-34, or SEQ ID NO: 38-40, or SEQ ID NO: 45-47;

And light chain variable region comprises CDRs with amino acid sequences of SEQ ID NO: 32-34, or SEQ ID NO: 38-40, or SEQ ID NO: 45-47;

Preferably, the CDRs in the light chain variable region and the heavy chain variable region are not the same.

Disclosed is an isolated nucleic acid molecule comprising a nucleotide sequence capable of encoding the heavy chain variable region of the antibody, wherein,

the heavy chain variable region of the said antibody comprises CDRs with amino acid sequences selected from SEQ ID NO: 29-31, SEQ ID NO: 35-37, or SEQ ID NO: 35, SEQ ID NO: 41, and SEQ ID NO: 37, or SEQ ID NO: 42-44;

and,

selected from SEQ ID NO: 32-34, or SEQ ID NO: 38-40, or SEQ ID NO: 45-47.

In another aspect, the present disclosure refers to a composition comprising isolated nucleic acid molecules encoding for the bispecific antibody as disclosed herein, wherein the composition comprises a first isolated nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain variable region of an antibody and a single-chain antibody, wherein

the heavy chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 29-31, and wherein the single-chain antibody comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35-37 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40; and a second isolated nucleic acid molecule comprising a nucleotide sequence encoding the light chain variable region of an antibody, wherein, the light chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 32-34.

In another aspect, the present disclosure refers to a composition comprising isolated nucleic acid molecules encoding for the bispecific antibody as disclosed herein, wherein the composition comprises a first isolated nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain variable region of an antibody and a single-chain antibody, wherein

the heavy chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 29-31,

and wherein the single-chain antibody comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35, 41 and 37 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40; and a second isolated nucleic acid molecule comprising a nucleotide sequence encoding the light chain variable region of an antibody, wherein, the light chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 32-34.

In another aspect, the present disclosure refers to a composition comprising isolated nucleic acid molecules encoding for the bispecific antibody as disclosed herein, wherein the composition comprises a first isolated nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain variable region of an antibody and a single-chain antibody, wherein

the heavy chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 29-31,

and wherein the single-chain antibody comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 42-44 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 45-47.

Disclosed is an isolated nucleic acid molecule comprising a nucleotide sequence capable of encoding the light chain variable region of the antibody, wherein,

the light chain variable region of the said antibody comprises CDRs with amino acid sequences selected from SEQ ID NO: 32-34, or SEQ ID NO: 38-40, or SEQ ID NO: 45-47; and a second isolated nucleic acid molecule comprising a nucleotide sequence encoding the light chain variable region of an antibody, wherein, the light chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 32-34.

In another example, the present disclosure refers to an isolated nucleic acid molecule comprising a nucleotide sequence encoding the light chain variable region of an antibody, wherein,

the light chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 32-34.

Disclosed is a vector comprising the isolated nucleic acid molecule described in the present disclosure.

In another example, the present disclosure refers to a vector, comprising the isolated nucleic acid molecule as described herein or any combination thereof.

Disclosed is a host cell comprising the isolated nucleic acid molecule described in the present disclosure, or the vector described in the present disclosure.

In another aspect, the present disclosure refers to a host cell line, comprising the composition as described herein.

Disclsoed is a method for preparing the bispecific antibodies described in the present disclosure, by culturing the host cells in the present disclosure under appropriate conditions, and recovering the said bispecific antibodies from the cell culture.

In another aspect, the present disclosure refers to a method for preparing the bispecific antibody as described herein, the method comprising culturing the host cell line as described herein under appropriate conditions, and recovering the bispecific antibody from the cell culture.

Disclosed are conjugates, including the bispecific antibodies described in the present disclosure and a conjugating partner as a detectable marker. Specifically, the said conjugating partners are radioactive isotopes, fluorescein, luminescent materials, colorful substances, or enzymes.

In another aspect, the present disclosure refers to a conjugate comprising the bispecific antibody as described herein and a conjugating partner as a detectable marker.

Disclosed are reagent kits, consisting of the bispecific antibodies or the conjugates described in the disclosure.

In another aspect, the present disclosure refers to a kit comprising the bispecific antibody as described herein, or the conjugate as described herein.

Specifically, the reagent kits may contain a secondary antibody, which specifically recognizes the said bispecific antibody; optionally, such secondary antibody may contain detectable markers such as radioactive isotopes, fluorescein, luminescent materials, colorful substances, or enzymes.

Disclosed is usage of the said bispecific antibodies described in the present disclosure to prepare reagent kits for detection of the existence or the levels of CTLA4 and/or PD-1 in samples.

In another example, there is provided use of the bispecific antibody as described herein in the preparation of a kit for the detection of the existence or the levels of CTLA4 and/or PD-1 in a sample.

Disclosed is a pharmaceutical composition comprising the said

bispecific antibodies or the conjugates described in the disclosure. Optionally, it may also comprise a pharmaceutically acceptable carrier or excipient.

In another aspect, the present disclosure refers to a pharmaceutical composition, comprising the bispecific antibody as described herein, or the conjugate as described herein.

Disclosed is use of the bispecific antibodies or conjugates described in the disclosure for producing drugs that are used for prevention and/or treatment of tumors or anemia, or for diagnosis of tumors or anemia; specifically, the said tumors may be melanoma, renal cancer, prostate cancer, bladder cancer, colorectal cancer, gastrointestinal cancer, liver cancer, non-small cell lung cancer, ovarian cancer and leukemia.

In another aspect, the present disclosure refers to use of the bispecific antibody as described herein, or the conjugate as described herein, or the pharmaceutical composition as described herein for the manufacture of a medicament for the treatment of a tumor.

In another example, the present disclosure refers to use of the bispecific antibody as disclosed herein, the conjugate as disclosed herein or the pharmaceutical composition as disclosed herein for the manufacture of a medicament for the treatment of anemia.

In another aspect, the present disclosure refers to use of the bispecific antibody as disclosed herein, the conjugate as disclosed herein or the pharmaceutical composition as disclosed herein for the manufacture of a medicament for the treatment of a viral or parasitic infection.

The present inventors have found through animal experiments that, bispecific antibody BiAb004 described in the disclosure can effectively inhibit the growth of MC38 tumor cells inoculated subcutaneously in right side of PD-1 HuGEMM mice, which the growth of tumor volume in PD-1 HuGEMM tumor-bearing mice is significantly inhibited.

Disclosed is the use of the bispecific antibodies or the conjugates described in the present disclosure, to prepare drugs with the following purposes:

Testing CTLA4 level in samples,

Blocking CTLA4 binding to B7,

Regulating (e.g. down-regulating) CTLA4 activity or CTLA4 levels,

Removing immunosuppression of CTLA4,

Activating T lymphocytes, or

Increasing the secretion of IL-2 in T lymphocytes;

And/or,

Blocking PD-1 binding to PDL1,

Regulating (e.g. down-regulating) PD-1 activity or PD-1 levels,

Removing immunosuppression of PD-1, or

Increasing the secretion of IFN-γ in T lymphocytes.

In another example, there is provided use of the bispecific antibody as described herein, or the conjugate as described herein, for the manufacturing of a medicament selected from the following:

a medicament for testing CTLA4 level in samples,

a medicament for blocking CTLA4 binding to B7,

a medicament for regulating (e.g. down-regulating) CTLA4 activity or CTLA4 levels,

a medicament for removing immunosuppression of CTLA4,

a medicament for activating T lymphocytes, or

a medicament for increasing the secretion of IL-2 in T lymphocytes;

And/or,

a medicament for blocking PD-1 binding to PDL1,

a medicament for regulating (e.g. down-regulating) PD-1 activity or PD-1 levels,

a medicament for removing immunosuppression of PD-1, or

a medicament for increasing the secretion of IFN-γ in T lymphocytes.

The present disclosure relates to an in vivo or in vitro method to apply to cells or subjects in need with an effective dose of the bispecific antibodies or the conjugates described in the present disclosure, and the said method is selected from the following:

Testing CTLA4 level in samples,

Blocking CTLA4 binding to B7,

Regulating (e.g. down-regulating) CTLA4 activity or CTLA4 levels,

Removing immunosuppression of CTLA4,

Activating T lymphocytes, or

Increasing the secretion of IL-2 in T lymphocytes;

And/or,

Blocking PD-1 binding to PDL1,

Regulating (e.g. down-regulating) PD-1 activity or PD-1 levels,

Removing immunosuppression of PD-1, or

Increasing the secretion of IFN-γ in T lymphocytes.

In another example, there is provided an in vivo or in vitro method, comprising administering to a cell or a subject in need with an effective dose of the bispecific antibody as described herein, or the conjugate as described herein, and for the purpose selected from the following:

Testing CTLA4 level in samples,

Blocking CTLA4 binding to B7,

Regulating (e.g. down-regulating) CTLA4 activity or CTLA4 levels,

Removing immunosuppression of CTLA4,

Activating T lymphocytes, or

Increasing the secretion of IL-2 in T lymphocytes;

And/or,

Blocking PD-1 binding to PDL1,

Regulating (e.g. down-regulating) PD-1 activity or PD-1 levels,

Removing immunosuppression of PD-1, or

Increasing the secretion of IFN-γ in T lymphocytes.

In in vitro experiments in the present disclosure, the anti-CTLA4 antibodies, the anti-PD-1 antibodies, and the anti-CTLA4-anti-PD-1 bispecific antibodies described in the present disclosure all can induce the secretion of IFN-γ, and activate the immune response.

Disclosed is a method for the prevention and/or treatment of tumors or anemia, or for diagnosis of tumors or anemia, including

procedures to apply to subjects in need with an effective dose of the bispecific antibodies or the conjugates described in the present disclosure; specifically, the said tumors may be melanoma, renal cancer, prostate cancer, bladder cancer, colorectal cancer, gastrointestinal cancer, liver cancer, non-small cell lung cancer, ovarian cancer or leukemia.

In another example, there is provided use of the bispecific antibody as described herein, or the conjugate as described herein, for the manufacturing of a medicament for the prevention, treatment, adjuvant treatment, and/or diagnosis of tumors or anemia in a subject in need thereof; preferably, the tumors may be melanoma, renal cancer, prostate cancer, bladder cancer, colorectal cancer, gastrointestinal cancer, liver cancer, non-small cell lung cancer, ovarian cancer or leukemia.

The use of bispecific antibodies or conjugates thereof described in the present disclosure for the prevention and/or treatment of tumors or anemia, or for diagnosis of tumors or anemia; specifically, the said tumors may be melanoma, renal cancer, prostate cancer, bladder cancer, colorectal cancer, gastrointestinal cancer, liver cancer, non-small cell lung cancer, ovarian cancer or leukemia.

Use of bispecific antibodies or the conjugates thereof described in the present disclosure, with the following purposes:

Blocking CTLA4 binding to B7,

Regulating (e.g. down-regulating) CTLA4 activity or CTLA4 levels,

Removing immunosuppression of CTLA4,

Activating T lymphocytes, or

Increasing the secretion of IL-2 in T lymphocytes;

And/or,

Blocking PD-1 binding to PDL1,

Regulating (e.g. down-regulating) PD-1 activity or PD-1 levels,

Removing immunosuppression of PD-1, or

Increasing the secretion of IFN-γ in T lymphocytes.

Antibody drugs, especially monoclonal antibodies (MAB), have shown good efficacy in the treatment of a variety of diseases. The traditional methods to obtain therapeutic antibodies are to immunize animals with antigens to generate antigen-specific antibodies, or to improve low affinity antibodies by affinity maturation. However, these methods are time- and effort-consuming, and often may not target the specific epitopes on the antigen.

The variable regions of light and heavy chains of antidodies determine binding of an antibody to its antigen; a variable region of each chain contains three highly variable regions, which are called complementarity determining region (CDR) (the CDRs of a heavy chain (H) consist of HCDR1, HCDR2 and HCDR3; the CDRs of a light chain (L) consist of LCDR1, LCDR2 and LCDR3; named by Kabat et al (Sequences of Proteins of Immunological Interest, Fifth Edition (1991), 1-3, NIH Publication 91-3242, Bethesda Md)).

Use conventional techniques known by those of ordinary skill in the art, for example, to analyze amino acid sequences of CDRs in the monoclonal antibodies listed in (1) - (13) through VBASE2 database, and the results are as follows:

(1) 14C12

The amino acid sequence of the heavy chain variable region is shown in SEQ ID NO: 16, and the amino acid sequence of the light chain variable region is shown in SEQ ID NO: 18.

The amino acid sequences of three CDRs of the heavy chain variable region are as follows:

HCDR1: GFAFSSYD (SEQ ID NO: 29)

HCDR2: ISGGGRYT (SEQ ID NO: 30)

HCDR3: ANRYGEAWFAY (SEQ ID NO: 31)

The amino acid sequences of three CDRs of the light chain variable region are as follows:

LCDR1: QDINTY (SEQ ID NO: 32)

LCDR2: RAN (SEQ ID NO: 33)

LCDR3: LQYDEFPLT (SEQ ID NO: 34)

(2) 14C12H1L1

The amino acid sequence of the heavy chain variable region is shown in SEQ ID NO: 20, and the amino acid sequence of the light chain variable region is shown in SEQ ID NO: 22.

The amino acid sequences of three CDRs of the heavy chain variable region are the same as those of 14C12.

The amino acid sequences of three CDRs of the light chain variable region are the same as those of 14C12.

(3) 4G10

The amino acid sequence of the heavy chain variable region is shown in SEQ ID NO: 2, and the amino acid sequence of the light chain variable region is shown in SEQ ID NO: 4.

The amino acid sequences of three CDRs of the heavy chain variable region are as follows:

HCDR1: GYSFTGYT (SEQ ID NO: 35)

HCDR2: INPYNNIT (SEQ ID NO: 36)

HCDR3: ARLDYRSY (SEQ ID NO: 37)

The amino acid sequences of three CDRs of the light chain variable region are as follows:

LCDR1: TGAVTTSNF (SEQ ID NO: 38)

LCDR2: GTN (SEQ ID NO: 39)

LCDR3: ALWYSNHWV (SEQ ID NO: 40)

(4) 4G10H1L1

The amino acid sequence of the heavy chain variable region is shown in SEQ ID NO: 6, and the amino acid sequence of the light chain variable region is shown in SEQ ID NO: 8.

The amino acid sequences of three CDRs of the heavy chain variable region are the same as those of 4G10.

The amino acid sequences of three CDRs of the light chain variable region are the same as those of 4G10.

(5) 4G10H3L3

The amino acid sequence of the heavy chain variable region is shown in SEQ ID NO: 10, and the amino acid sequence of the light chain variable region is shown in SEQ ID NO: 12.

The amino acid sequences of three CDRs of the heavy chain variable region are the same as those of 4G10.

The amino acid sequences of three CDRs of the light chain variable region are the same as those of 4G10.

(6) 4G10H4L3

The amino acid sequence of the heavy chain variable region is shown in SEQ ID NO: 14, and the amino acid sequence of the light chain variable region is shown in SEQ ID NO: 12.

The amino acid sequences of three CDRs of the heavy chain variable region are as follows:

HCDR1: GYSFTGYT (SEQ ID NO: 35)

HCDR2: INPYNDIT (SEQ ID NO: 41)

HCDR3: ARLDYRSY (SEQ ID NO: 37)

The amino acid sequences of three CDRs of the light chain variable region are the same as those of 4G10.

(7) 8D2H14L2

The amino acid sequence of the heavy chain variable region is shown in SEQ ID NO: 25, and the amino acid sequence of the light chain variable region is shown in SEQ ID

NO: 27.

The amino acid sequences of three CDRs of the heavy chain variable region are as follows:

HCDR1: GFTFSDNW (SEQ ID NO: 42)

HCDR2: IRNKPYNYET (SEQ ID NO: 43)

HCDR3: TAQFAY (SEQ ID NO: 44)

The amino acid sequences of three CDRs of the light chain variable region are as follows:

LCDR1: ENIYGG (SEQ ID NO: 45)

LCDR2: GAT (SEQ ID NO: 46)

LCDR3: QNVLRSPFTF (SEQ ID NO: 47)

(8) BiAb001

The amino acid sequences of nine CDRs of the heavy chain variable regions are as follows

HCDR1: GFAFSSYD (SEQ ID NO: 29)

HCDR2: ISGGGRYT (SEQ ID NO: 30)

HCDR3: ANRYGEAWFAY (SEQ ID NO: 31)

HCDR4: GYSFTGYT (SEQ ID NO: 35)

HCDR5: INPYNNIT (SEQ ID NO: 36)

HCDR6: ARLDYRSY (SEQ ID NO: 37)

HCDR7: TGAVTTSNF (SEQ ID NO: 38)

HCDR8: GTN (SEQ ID NO: 39)

HCDR9: ALWYSNHWV (SEQ ID NO: 40)

The amino acid sequences of three CDRs of the light chain variable region are as follows:

LCDR1: QDINTY (SEQ ID NO: 32)

LCDR2: RAN (SEQ ID NO: 33)

LCDR3: LQYDEFPLT (SEQ ID NO: 34)

(9) BiAb002

The amino acid sequences of nine CDRs of the heavy chain variable regions are the

same as those of BiAb001.

The amino acid sequences of three CDRs of the light chain variable region are the same as those of BiAb001.

(10) BiAb003

The amino acid sequences of nine CDRs of the heavy chain variable regions are the same as those of BiAb001.

The amino acid sequences of three CDRs of the light chain variable region are the same as those of BiAb001.

(11) BiAb004

The amino acid sequences of nine CDRs of the heavy chain variable regions are the same as those of BiAb001.

The amino acid sequences of three CDRs of the light chain variable region are the same as those of BiAb001.

(12) BiAb007

The amino acid sequences of nine CDRs of the heavy chain variable regions are as follows:

HCDR1: GFAFSSYD (SEQ ID NO: 29)

HCDR2: ISGGGRYT (SEQ ID NO: 30)

HCDR3: ANRYGEAWFAY (SEQ ID NO: 31)

HCDR4: GYSFTGYT (SEQ ID NO: 35)

HCDR5: INPYNDIT (SEQ ID NO: 41)

HCDR6: ARLDYRSY (SEQ ID NO: 37)

HCDR7: TGAVTTSNF (SEQ ID NO: 38)

HCDR8: GTN (SEQ ID NO: 39)

HCDR9: ALWYSNHWV (SEQ ID NO: 40)

The amino acid sequences of three CDRs of the light chain variable region are the same as those of BiAb001.

(13) BiAb010

The amino acid sequences of nine CDRs of the heavy chain variable regions are as follows:

HCDR1: GFAFSSYD (SEQ ID NO: 29)

HCDR2: ISGGGRYT (SEQ ID NO: 30)

HCDR3: ANRYGEAWFAY (SEQ ID NO: 31)

HCDR4: GFTFSDNW (SEQ ID NO: 42)

HCDR5: IRNKPYNYET (SEQ ID NO: 43)

HCDR6: TAQFAY (SEQ ID NO: 44)

HCDR7: ENIYGG (SEQ ID NO: 45)

HCDR8: GAT (SEQ ID NO: 46)

HCDR9: QNVLRSPFTF (SEQ ID NO: 47)

The amino acid sequences of three CDRs of the light chain variable region are the same as those of BiAb001.

Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Furthermore, laboratory techniques of cell and tissue culture, molecular genetics, oligo- or polynucleotide chemistry, and immunology described herein are those well-known and commonly used in the art. Meanwhile, to better understand the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

As used herein, the term ”amino acid sequence of CTLA4 (Cytotoxic T-Lymphocyte Antigen 4)” refers to not only the full-length CTLA4 protein, but also, the extracellular fragment of CTLA4 (CTLA4ECD), or fragments containing CTLA4ECD, or fusion proteins of CTLA4ECD, such as fragments of fusions with mouse or human IgG Fc fragments (mFc or hFc). However, understood by those of ordinary skill in the art, the amino acid sequence of CTLA4 protein can have natural or artificial mutations or variation (including but not limited to substitutions, deletions, and/or additions), not affecting its biological functions. Thus, in the present disclosure, the term “CTLA4 protein” also includes these amino acid sequences containing natural or artificial variants. Additionally, when referring to sequence fragments of CTLA4 protein, the sequence fragments containing natural or artificial variants are also included.

As used herein, the term “amino acid sequence of PD-1 (Programmed cell death protein 1, NCBI GenBank: 005018 NM)” refers to not only the full-length PD-1 protein, but also, the extracellular fragment of PD-1, PD-1ECD), or fragments containing PD-1ECD, or fusion proteins of PD-1ECD, such as fragments of fusions with mouse or human IgG Fc fragments (mFc or hFc). However, understood by those of ordinary skill in the art, the amino acid sequence of PD-1 protein can have natural or artificial mutations or variation (including but not limited to substitutions, deletions, and/or additions), not affecting its biological functions. Thus, in the present disclosure, the term “PD-1 protein” also includes these amino acid sequences containing natural or artificial variants. Additionally, when referring to sequence fragments of PD-1 protein, the sequences fragments containing natural or artificial variants are also included.

As used in this disclosure, if not specifically stated, B7 protein described herein is B7-1 and/or B7-2 protein whose amino acid sequences are well known in the prior art, which can be referenced from the existing literature or sequences disclosed in GenBank. For example, B7-1 (CD80, NCBI Gene ID: 941) and B7-2 (CD86, NCBI Gene ID: 942).

As used herein, the term “EC_{50}” refers to the concentration of 50% of maximal effect.

As used herein, the term "antibody" refers to an immunoglobulin molecule normally composed of two pairs of peptides (each pair with a "light" (L) chain and a "heavy" (H) chain). In general, the heavy chain can be comprehended as the polypeptide chain with a higher molecular weight, while the light chain refers to the polypeptide chain with a lower molecular weight. The light chains of an antibody are classified as either κ or λ light chains, while the heavy chains of an antibody are classified as μ, δ, γ, α or ε heavy chains, which define the antibody isotypes as IgM, IgD, IgG, IgA, and IgE, respectively.

Within a light and a heavy chain, the variable region and the constant region are connected through a "J" region consisting of about 12 or more amino acids, and a heavy chain also contains a "D" region consisting of about three or more amino acids. A heavy chain consists of a heavy chain variable region (V_H) and a heavy chain constant region (C_H). A heavy chain constant region consists of three structural domains (C_H1, C_H2, and

C_H3). A light chain consists of a light chain variable region (V_L) and a light chain constant region (C_L). The constant region of a light chain consists of a structural domain C_L. The constant region of an antibody mediates the binding of an immunoglobulin to host tissues or factors, including various immune cells (e.g. effector cells) and the complement component 1q (C1q) of the classical complement system. V_H and V_L regions can further be subdivided into regions with high variability (known as complementarity determining region (CDR)), separated by relatively conservative regions called framework region (FR). Each V_H and V_L comprises three CDRs and four FRs in the order of FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4 from the amino terminus to the carboxyl terminus. The variable regions (V_H and V_L) of each heavy/light chain form the antibody binding sites respectively. Distribution of amino acids to the regions or domains follows the definitions by Kabat Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md.) (1987 and 1991), or Chothia & Lesk (1987) j. Mol. Biol. 196:901-917; Chothia et al. (1989) Nature 342:878-883. In particular, heavy chains can also contain more than three CDRs, such as 6, 9, or 12. For example, in the case of the bispecific antibodies in the present disclosure, the heavy chain can be a heavy chain of an IgG antibody with a scFv of another antibody connected to its C terminus, thus this heavy chain contains 9 CDRs. The term "antibody" is not restricted by any particular method of making antibodies. For example, it includes, in particular, recombinant antibodies, monoclonal antibodies or polyclonal antibodies. Antibodies can be of different isoforms, such as IgG (for example, IgG1, IgG2, IgG3 or IgG4 subtypes), IgA1, IgA2, IgD, IgE or IgM antibodies.

As used herein, the term "antigen-binding fragments" refers to polypeptides containing fragments of a full-length antibody, maintaining the ability to bind specifically to the same antigen, and/or to compete with the full length antibody against the antigen, which is also called “the antigen binding portion". See Fundamental Immunology, Ch. 7 (Paul, W., 2nd edition, Raven Press, N.Y. (1989)), including the entire article and references in this disclosure for all purposes. Antigen-binding fragments can be produced by recombinant DNA techniques or by cleaving intact antibodies with proteolytic enzymes or chemicals. In some cases, the antigen-binding fragments include Fab, Fab',

F(ab')2, Fd, Fv, dAb and CDR fragments, single-chain antibodies (e.g. scFV), chimeric antibodies, diabody, and polypeptide which includes at least a portion of the antibody which is sufficient to confer a specific antigen binding capacity.

As used herein the term "Fd fragment" refers to an antibody fragment composed of V_H and C_H1 domains. The term "Fv fragment" refers to an antibody fragment composed of the V_L and V_H domains from a single arm of the antibody. The term "dAb fragment" refers to an antibody fragment composed of a V_H domain (Ward et al., Nature 341:544 546 (1989)). The term "Fab fragment" refers to an antibody fragment composed of V_L, V_H, C_L and C_H1 domains. The term "F(ab')2 fragment" refers to an antibody fragment containing two Fab fragments connected by a disulfide bridge in the hinge region.

In some cases, the antigen-binding fragments of an antibody are single-chain antibodies (e.g. scFv), a single polypeptide chain composed of V_L and V_H domains linked together (see, for example, Bird et al., Science 242: 423-426 (1988) and Huston et al., Proc. Natl. Acad. Sci. USA 85: 5879- 5883 (1988)). Such scFv molecules may have a common structure: NH_2-V_L-linker-V_H-COOH or NH_2-V_H-linker-V_L-COOH. The appropriate linker may be a repeat of GGGGS or its variants, for example, amino acid sequence of (GGGGS)4 or its variants (Holliger et al., (1993), Proc. Natl. Acad. Sci. USA 90: 6444-6448). Other applicable linkers had been described by Alfthan, et al., (1995), Protein Eng. 8: 725-731, Choi, et al., (2001) Eur. J. Immunol. 31: 94-106, Hu, et al., (1996), Cancer Res. 56: 3055-3061, Kipriyanov et al., (1999), J. Mol. Biol. 293: 41-56 and Roovers, et al., (2001) Cancer Immunol.

In some cases, the antigen binding fragment is a diabody, namely, a dimeric antibody, whose V_H and V_L domains are lined on a single polypeptide chain, while because of the the too short linker to allow pairing between the two domains of on same chain, thus the domains are forced to pair with complementary domains on another chain to generate two antigen binding sites (see, for example, Holliger P. et al., Proc. Natl. Acad. Sci. USA 90: 6444-6448 (1993), and Poljak R. J. et al., Structure 2: 1121-1123 (1994))..

Using conventional techniques known by those of ordinary skill in the art (such as recombinant DNA technology or enzymatic/chemical cleavage), an antigen binding fragment (such as the antibody fragments described above) may be obtained from a given

antibody, and screened for specificity in the same manner as for the full antibody.

In the present disclosure, unless specified otherwise, the term "antibody" refers to not only the intact antibody, but also the antigen binding fragments of the antibody.

As used in this disclosure, the terms "mAb" and "monoclonal antibodies" refers to an antibody or a fragment of an antibody that is derived from a group of highly homologous antibodies, i.e. from a group of identical antibody molecules, except for mutations that may arise spontaneously. Monoclonal antibody has high specificity against a single epitope on the antigen. Polyclonal antibodies are different from monoclonal antibodies, containing at least 2 or more different antibodies, which usually recognize different epitopes on the antigen. Monoclonal antibodies can be obtained with hybridoma technology reported originally by Kohler et al., (Nature, 256: 495, (1975)), as well as recombinant DNA Technology (see U.S. Patent 4,816,567).

As used in this disclosure, the term "chimeric antibody" refers to an antibody in which parts of the light chain and/or heavy chain are from one antibody (can be from a particular species or belong to a specific antibody class or subclass), and the other parts of the light chain and/or heavy chain are from another antibody (can be from the same or different species or belong to the same or different antibodies classes or subclass). Nevertheless, it retains antigen binding activity (U.S.P to Cabilly et al., 4816567; Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851 6855 (1984)).

As used in this disclosure, the term "humanized antibody" refers to an antibody or its fragments, derived from a human immunoglobulin (receptor antibody), whose CDRs or parts of CDRs are replaced by CDRs from a non-human antibody (donor antibody), where the donor antibody may be a non-human antibody (for example, from mice, rats, or rabbits) with predictable specificity, binding affinity, and reactivity. In addition, to further improve or optimize the performance of the antibody, some amino acid residues in framework regions (FR) of the receptor antibody can also be replaced by the corresponding amino acid residues of non-human species, or replaced by the corresponding amino acid residues of other antibodies. For more details on humanized antibodies, see for example Jones, et al., Nature, 321: 522-525 (1986); Reichmann et al., Nature, 332: 323-329 (1988); Presta, Curr. Op. Struct. Biol., 2: 593-596 (1992); and

Clark, Immunol. Today, 21: 397-402 (2000).

As used in this disclosure, the term "Epitope" refers to a site on the antigen that the immunoglobulin or antibody can specifically bind to. "Epitope" is also known as the "antigenic determinant" in this field. Epitope or antigenic determinants usually consist of chemically active surface groups of molecules, such as amino acids, carbohydrates or glycoside chains, and usually have specific three dimensional structures, as well as specific charge characteristics. For example, epitopes typically consist of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 consecutive or non-consecutive amino acids in a unique spatial conformation, which can be "linear" or "conformational". See, for example, Epitope Mapping Protocols in Methods in Molecular Biology, volume 66, G. E. Morris, Ed. (1996). In a linear epitope, the interacting points between the protein and interacting molecule (e.g., antibodies) exist linearly along the primary amino acid sequence; while in a conformational epitope, the interacting points are separated along the primary amino acid sequence.

As used in this disclosure, the term "isolate" or "isolated" refers to obtained by artificial means in the natural state. If there is an "isolated" substance or component in nature, it may be due to the change in its natural environment, or isolated from the natural environment, or both. For example, polynucleotide or polypeptide in a natural existence in a living animal will be called “isolated” if it was separated with high purity in the same natural state. The term "isolate" or "isolated" does not exclude existence of artificial or synthetic material, or other impurities that does not affect the activity.

As used in this disclosure, the term "*E. coli* expression system" refers to the expression system composed of *Escherichia coli* (strain) and vector, where *E. coli* (strain) is commercially available, including but not limited to: GI698, ER2566, BL21 (DE3), B834 (DE3), and BLR (DE3).

As used in this disclosure, the term "vector" refers to a nucleic acid delivery vehicle that can be inserted with a polynucleotide. The vector that can have the protein that is encoded by the inserted polynucleotide expressed is called an expression vector. Vectors can be inserted into the host cell by transformation, transduction, or transfection, so that the genetic substances carried by the vector can be expressed in the host cell. Vectors are

well known to the technical personnel in the field, including but not limited to: plasmid; phasmid; cosmid; artificial chromosome such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or P1 derived artificial chromosome (PAC); phage such as λ phage or M13 phage and animal viruses etc. Animal viruses may include but not limited to, reverse transcriptase virus (including lentivirus), adenovirus, adeno-associated virus, herpes virus (e. g. herpes simplex virus), chicken pox virus, baculovirus, papilloma virus, and papova virus (such as SV40). A vector can contain multiple components that control expression, including but not limited to, promoter, transcription initiation factor, enhancer, selection element, and reporter gene. In addition, the vector may also contain replication initiation site.

As used in this disclosure, the term "host cell" refers to cells that can import vectors, including but not limited to, prokaryotic cells such as *E. coli* and *Bacillus subtilis*, fungal cells such as yeast and *Aspergillus*, insect cells such as S2 drosophila cells and Sf9, or animal cells such as fibroblast cells, CHO cells, COS cells, NSO cells, HeLa cells, BHK cells, HEK293 cells or human cells.

As used in this disclosure, "Homologous" refers to the sequence similarity or sequence identity between two polypeptides or between two nucleic acid molecules. When a position in the two compared sequences is occupied by the same base or amino acid, e.g., if a position in two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The “percent homology” of two sequences is calculated by the function of the numbers of matched positions of the two sequences divided by the total numbers of positions that are compared multiplied by 100. For example, if 6 out of 10 positions of two sequences are matched, thus the homology of the two squences is 60%. For example, homology of DNA sequences CTGACT and CAGGTT is 50% (3 out of 6 positions are matched). Generally, a comparison is made when two sequences are aligned to give maximum identity. Such alignment can be simply generated using computer programs, for instance, the ALIGN program (DNAstar, Inc.), implemented by the method of Needleman et al. (1970) J. Mol. Biol. 48: 443-453. Or, using the algorithm proposed by E. Meyers and W. Miller (Comput. Appl Biosci. , 4:11-17 (1988)) that has been integrated into ALIGN program (version 2.0), in whcih the percent homology of

two sequences is calculated by using PAM120 residue weight table, a gap length penalty of 12 and gap penalty of 4. In addition, GAP program that has been integrated into the GCG software package (available on www.gcg.com) implemented with algorithm of Needleman and Wunsch (J MoI Biol. 4-453 (1970)), Blossum 62 matrix, PAM250 matrix as well as 16, 14, 12, 10, 8, 6 or 4 GAP weight and 1, 2, 3, 4, 5 or 6 length weight can be used to measure the percent homology of two amino acid sequences.

As used in this disclosure, the term “specific binding” refers to non-randomly binding between two molecules, i.e., interaction between antibodies and antigen. In some examples, the antibody specificly binding to the antigen (or antibody with specifity to an antigen) refers that the antibody binds the antigen with an affinity (K_D) smaller approximately than 10^{-5} M, such as smaller than 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M, 10^{-10} M or even less. In some examples of the disclosure, the term “target(ed)” refers to specific binding.

As used in this disclosure, the term "K_D" refers to the dissociation equilibrium constant for specific antibody-antigen interactions, to describe the binding affinity between antibodies and antigens. The smaller the equilibrium dissociation constant is, the tighter the antibody binds antigen, the higher the affinity between the antibody and the antigen is. Generally, antibodies bind antigens with a dissociation equilibrium constant (K_D) less than approximately 10^{-5} M, in particular, less than approximately 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M, or 10^{-10} M, or less, for example, as measured with a BIACORE instrument by surface plasmon resonance (SPR).

As used in this disclosure, the terms "monoclonal antibodies" and "mAb" have the same meaning and are used interchangeably; the terms "polyclonal antibodies" and "PcAb" have the same meaning and are used interchangeably; the terms "polypeptide" and "protein" have the same meaning and are used interchangeably. Also in the present disclosure, amino acids are usually represented by single letter or three letter abbreviations known in the field. For example, alanine can be represented by A or Ala.

As used in this disclosure, the terms "hybridoma" and "hybridoma cell line" are used interchangeably, and when the terms "hybridoma" and "hybridoma cell line" are used, they also include subclones and progenies of the hybridoma cell line. For example, when

referring to the hybridoma cell lines LT002 or LT003, it also refers to the subclones and progenies of the hybridoma cell lines LT002 or LT003.

As used in this disclosure, the term "pharmaceutically acceptable carrier and/or excipient" refers to a carrier and/or excipient that is compatible with the subject and active ingredients in pharmacology and/or physiology and is known to this field (e.g. Remington's Pharmaceutical Sciences. Edited by Gennaro AR, 19th ed. Pennsylvania: Mack Publishing Company,1995), including but not limited to: pH regulator, surfactant, adjuvant, and ionic strength enhancer. For example, pH regulators include but not limited to phosphate buffers; Surfactants include but not limited to cations, anions or non-ionic surfactants such as Tween 80; Ionic strength enhancers include but not limited to sodium chloride.

As used in this disclosure, the term "adjuvant" refers to a nonspecific immune booster that, when delivered into the body together or beforehand with an antigen enhances or changes the body's immune response to the antigen. There are many kinds of adjuvants, including but not limited to aluminum adjuvants (such as aluminum hydroxide), freund's adjuvants (such as complete and incomplete freund's adjuvants), corynebacterium parvum, lipopolysaccharides, cytokines, etc. Freund's adjuvant is currently the most commonly used adjuvant in animal experiments. Aluminum hydroxide adjuvant is used mostly in clinical trials.

As used in this disclosure, the term "effective dose" refers to the quantity that is sufficient to partially or completely achieve the desired effect. For example, effective prevention dose (e.g., diseases associated with CTLA4 binding to B7 or hyperactivity of CTLA4, such as tumors) is defined as the amount of a therapeutic sufficient to prevent, stop, or delay the diseases (e.g., diseases associated with hyperactivity of CTLA4 binding to B7 or diseases associated with hyperactivity of CTLA4, such as tumors); effective treatment dose is the amount of a therapeutic to cure, or at least partially stop, the disease and its complications in sick patients. Determination of such an effective dose is entirely within the scope of the capabilities of the technical personnel in the field. For example, the effective treatment dose will depend on the severity of the disease, the overall state of the patient's own immune system, the general background of patients such as age, weight

and sex, administration of drugs, and other treatments at the same time.

Effects of the Disclosure

The monoclonal antibodies in the present disclosure, 4G10H1L1 and 4G10H3L3, are capable of binding to CTLA4 specifically, effectively blocking the interaction of CLTA4 and B7, and removing the immunosuppression of CLTA4 specifically to activate T lymphocytes.

The monoclonal antibody 14C12H1L1 is capable of binding to CTLA4 specifically, effectively blocking the interaction of CTLA4 and B7, and removing the immunosuppression of CTLA4 specifically to activate T lymphocytes.

The bispecific antibodies of the present disclosure have the potential to be prepared for drugs for the prevention and/or treatment of tumors, such as melanoma, renal cancer, prostate cancer, bladder cancer, colorectal cancer, gastrointestinal cancer, liver cancer, non-small cell lung cancer, ovarian cancer and leukemia.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 SDS-PAGE Results of Monoclonal Antibody 4G10. From left to right: 1 μg antibody in non-reduced loading buffer; 1 μg antibody in reduced loading buffer; 5 μL Marker; 1 μg BSA.

Figure 2 SDS-PAGE Results of Monoclonal Antibody 4G10H1L1. From left to right: 1 μg antibody in non-reduced loading buffer; 1 μg antibody in reduced loading buffer; 5 μL Marker.

Figure 3 SDS-PAGE Results of Monoclonal Antibody 4G10H3L3. From left to right: 1 μg antibody in reduced loading buffer; 5 μL Marker.

Figure 4 SDS-PAGE Results of Monoclonal Antibody 14C12H1L1. From left to right: 1 μg antibody in non-reduced loading buffer; 1 μg antibody in reduced loading buffer; 5 μL Marker; 1 μg BSA.

Figure 5 SDS-PAGE Results of Bispecific Antibody BiAb001. From left to right: 5 μL Marker; 1 μg antibody in non-reduced loading buffer; 1 μg antibody in reduced loading buffer; 1 μg BSA.

Figure 6 SDS-PAGE Results of Bispecific Antibody BiAb002. From left to right: 5 μL Marker; 1 μg antibody in non-reduced loading buffer; 1 μg antibody in reduced loading buffer; 1 μg BSA.

Figure 7 SDS-PAGE Results of Bispecific Antibody BiAb003. From left to right: 5 μL Marker; 1 μg antibody in non-reduced loading buffer; 1 μg antibody in reduced loading buffer; 1 μg BSA.

Figure 8 SDS-PAGE Results of Bispecific Antibody BiAb004. From left to right: 5 μL Marker; 1 μg antibody in non-reduced loading buffer; 1 μg antibody in reduced loading buffer; 1 μg BSA.

Figure 9 SDS-PAGE Results of Bispecific Antibody BiAb007. From left to right: 5 μL Marker; 1 μg antibody in non-reduced loading buffer; 1 μg antibody in reduced loading buffer; 1 μg BSA.

Figure 10 SDS-PAGE Results of Bispecific Antibody BiAb010. From left to right: 5 μL Marker; 1 μg antibody in non-reduced loading buffer; 1 μg antibody in reduced loading buffer; 1 μg BSA.

Figure 11. Binding kinetics of antibody 4G10

Figure 12. Binding kinetics of antibody 4G10 H1L1

Figure 13. Binding kinetics of antibody 4G10H3L3

Figure 14. Binding kinetics of antibody 4G10H4L3

Figure 15. Binding kinetics of antibody14C12

Figure 16. Binding kinetics of antibody14C12 H1L1

Figure 17. Binding kinetics of CTLA4 and antibody BiAb001

Figure 18. Binding kinetics of CTLA4 and antibody BiAb002

Figure 19. Binding kinetics of CTLA4 and antibody BiAb003

Figure 20. Binding kinetics of CTLA4 and antibody BiAb004

Figure 21. Binding kinetics of CTLA4 and antibody BiAb007

Figure 22. Binding kinetics of PD-1 and antibody BiAb001

Figure 23. Binding kinetics of PD-1 and antibody BiAb002

Figure 24. Binding kinetics of PD-1 and antibody BiAb003

Figure 25. Binding kinetics of PD-1 and antibody BiAb004

Figure 26. Binding kinetics of PD-1 and antibody BiAb007

Figure 27. Binding kinetics of PD-1 and antibody BiAb010

Figure 28. Indirect ELISA results of 4G10H1L1 and 4G10H3L3 binding to CTLA4.

Figure 29. Competition ELISA results of 4G10H1L1 and 4G10H3L3 binding to CTLA4 against B7.

Figure 30. Indirect ELISA results of 14C12 and 14C12H1L1 binding to PD-1.

Figure 31. Competition ELISA results of 14C12 and 14C12H1L1 binding to PD-1 against PDL1.

Figure 32. Indirect ELISA results of BiAb001, BiAb002, BiAb003, and BiAb004 binding to CTLA4.

Figure 33. Indirect ELISA results of BiAb001, BiAb002, BiAb003, and BiAb004 binding to PD-1.

Figure 34. Competition ELISA results of BiAb001, BiAb002, BiAb003, and BiAb004 binding to CTLA4 against B7.

Figure 35. Competition ELISA results of BiAb001, BiAb002, BiAb003, and BiAb004 binding to PD-1 against PDL1.

Figure 36. EC50 of 4G10H1L1 binding to CTLA4 on the Surface of 293T-CTLA4 Cells.

Figure 37. EC50 of 4G10H3L3 binding to CTLA4 on the Surface of 293T-CTLA4 Cells.

Figure 38. EC50 of 14C12H1L1 binding to PD-1 on the Surface of 293T-PD-1 Cells.

Figure 39. EC50 of BiAb001 binding to CTLA4 on the Surface of 293T-CTLA4 Cells.

Figure 40. EC50 of BiAb002 binding to CTLA4 on the Surface of 293T-CTLA4 Cells.

Figure 41. EC50 of BiAb003 binding to CTLA4 on the Surface of 293T-CTLA4 Cells.

Figure 42. EC50 of BiAb004 binding to CTLA4 on the Surface of 293T-CTLA4 Cells.

Figure 43. EC50 of BiAb001 binding to PD-1 on the Surface of 293T-PD-1 Cells.

Figure 44. EC50 of BiAb002 binding to PD-1 on the Surface of 293T-PD-1 Cells.

Figure 45. EC50 of BiAb003 binding to PD-1 on the Surface of 293T-PD-1 Cells.

Figure 46. EC50 of BiAb004 binding to PD-1 on the Surface of 293T-PD-1 Cells.

Figure 47. Binding activity of 4G10H3L3 to T Cell Surface Antigen CTLA4.

Figure 48. Binding activity of 14C12H1L1 to T Cell Surface Antigen PD-1.

Figure 49. Binding activity of BiAb003 and BiAb004 to T Cell Surface Antigens compared with those of 14C12H1L1and 4G10H3L3.

Figure 50. Effects of 4G10H1L1 and 4G10H3L3 on IFN-γ Secretion of Mixed Lymphocytes.

Figure 51. Effect of 14C12H1L1 on IFN-γ Secretion of Mixed Lymphocytes.

Figure 52. Effects of BiAb001 and BiAb002 on IFN-γ Secretion of Mixed Lymphocytes compared with those of 14C12H1L1 and 4G10H1L1.

Figure 53. Effects of BiAb003 and BiAb004 on IFN-γ Secretion of Mixed Lymphocytes compared with effects of 14C12H1L1 and 4G10H3L3.

Figure 54. Effect of 4G10H3L3 on IL-2 Secretion of Mixed Lymphocytes.

Figure 55. Effect of 14C12H1L1 on IL-2 Secretion of Mixed Lymphocytes.

Figure 56. Effects of BiAb003 and BiAb004 on IL-2 Secretion of Mixed Lymphocytes compared with those of 14C12H1L1 and 4G10H3L3.

Figure 57. Effects of 4G10H1L1 and 4G10H3L3 on IL-2 Secretion induced by co-culturing of PBMC, MDA-MB-231 and Raji cells.

Figure 58. Effect of 14C12H1L1 on IL-2 Secretion induced by co-culturing of PBMC, MDA-MB-231 and Raji cells.

Figure 59. Effect of BiAb001, BiAb002, BiAb003, and BiAb004 on IL-2 Secretion induced by co-culturing of PBMC, MDA-MB-231 and Raji cells, compared with those of 4G10H1L1, 4G10H3L3, and 14C12H1L1.

Figure 60. Effect of BiAb004 on the Tumor Growth of MC38 Tumor Model in PD-1 HuGEMM Mice.

Description of the deposited biological materials

LT002 (CTLA4-4G10), a hybridoma cell line, was preserved in China Center for

Type Culture Collection (CCTCC) on June 16, 2015. Deposit Accession NO. : C201587, Depository address: Wuhan university, Wuhan, China, zip code: 430072.

LT003 (PD-1-14C12), a hybridoma cell line, was preserved in China Center for Type Culture Collection (CCTCC) on June 16, 2015. Deposit Accession NO. : C2015105, Depository address: Wuhan university, Wuhan, China, zip code: 430072.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail. As will be appreciated by one skilled in the art, the following examples are only used for the description of the invention, and not to be deemed to limit the scope of the invention. The cases without the specific descriptions of techniques or conditions were carried out in accordance with the literature in the field (e.g., Guide to Molecular Cloning, written by J Sambrook, et al, translated by Peitang Huang, et al, third Edition, Science Press) or in accordance with the product instruction manual. The reagents or instruments with no specified manufacturer were all conventional products available commercially.

In the embodimts of the present invention, the T cells used were from Akeso Biopharma, Inc., the BALB/C mice were purchased from the Guangdong Medical Laboratory Animal Center. The PD-1 HuGEMM mice used were from Nanjing Galaxy Biopharma Co., Ltd.; MC38 cells were from Shanghai Fudan IBS Cell Center.

Example 1: Preparation of anti-CTLA4 antibody 4G10

1. Establishment of hybridoma cell line LT002

Using CTLA4-mFc (a fusion protein of human CTLA4 protein (GenbankID: NP 005205.2) extracellular region and mouse IgG1Fc protein) as the antigen, the hybridoma cells were obtained by fusing the splenocytes of immunized BALB/C mice (purchased from Guangdong Medical Laboratory Animal Center) and mouse myeloma cells with currently established method (for example, Stewart, S.J., "Monoclonal Antibody Production", in Basic Methods in antibody Production and Characterization, Eds.G.C. Howard and D.R. Bethell, Boca Raton: CRC Press, 2000).

The CTLA4 protein was generated by digesting the fusion protein CTLA4-mFc with

TEV protease, and further purified by purification column. Microplatewas coated with CTLA4 as the antigen, and the above hybridoma cells were screened by indirect ELISA to select those secreting new antibodies specifically binding to CTLA4. The hybridoma cells screened via indirect ELISA were further screened by competitive ELISA against ligand B7-1 (CD80, NCBI Gene ID: 941) and B7-2 (CD86, NCBI Gene ID: 942) to select those secreting monoclonal antibodies that competitively bind to CTLA4, and then a stable hybridoma cell line was obtained by limited dilution method. This hybridoma cell line was named LT002 (CTLA4-4G10), and its secreted monoclonal antibody is named 4G10.

LT002 (CTLA4-4G10), the hybridoma cell line, was preserved in China Center for Type Culture Collection (CCTCC) on June 16, 2015. Deposit Accession NO.: C201587, Depository address: Wuhan university, Wuhan, China, postcode: 430072.

2. Preparation of anti-CTLA4 antibody 4G10

The LT002 cells in the present invention were cultured using IMDM medium containing 10% low IgG fetal bovine serum (IMDM medium containing 1% streptomycin, cultured in cell incubator with 5% CO_2, 37°C), and then the cell culture supernatant was harvested and purified by high-speed centrifugation after 7 days culture, filtration through microporous membrane, and HiTrap protein A HP column to get the antibody 4G10. The purified 4G10 were identified on SDS-PAGE electrophoresis, and the result was shown in Figure 1.

Example 2: Sequence analysis of anti-CTLA4 antibody 4G10

Sequence analysis of antibody 4G10

mRNA was extracted from the hybridoma cell line LT002 prepared in Example 1 above according to the manual of the cell/bacterial total RNA extraction reagent kit (Tiangen, Product No DP430).

cDNA was synthesized using Invitrogen SuperScript ® III First - Strand Synthesis System for RT-PCR, and amplified by PCR.

TA cloning was directly carried out on the PCR amplified product according to the instructions of pEASY-T1 Cloning Kit (Transgen CT101).

The products of TA cloning were directly sequenced, and the sequencing results were as follows:

Nucleic acid sequence of heavy chain variable region: (372 bp)

CAGGTCAAGCTGCAGGAGTCTGGACCTGAGCTGGTGAAGCCTGGAGCTT
CAATGAAGATATCCTGCAAGGCTTCTGGTTACTCATTCACTGGCTACACCATGA
ACTGGGTGAAGCAGAGCCATGGAAAGAACCTTGAATGGATTGGACTTATTAAT
CCTTACAATAATATTACTAACTACAACCAGAAGTTCATGGGCAAGGCCACATTT
ACTGTAGACAAGTCATCCAGCACAGCCTACATGGAACTCCTCAGACTGACATC
TGAAGACTCTGGAGTCTATTTCTGTGCAAGACTCGACTATAGGTCTTATTGGGG
CCAAGGGACTCTGGTCACTGTCTCTGCAGCCAAAACGACACCCCCATCTGTCT
AT (SEQ ID NO: 1)

Encoded amino acid sequence: (124 aa)

QVKLQESGPELVKPGASMKISCKASGYSFTGYTMNWVKQSHGKNLEWIGLI
NPYNNITNYNQKFMGKATFTVDKSSSTAYMELLRLTSEDSGVYFCARLDYRSYW
GQGTLVTVSAAKTTPPSVY (SEQ ID NO: 2)

Nucleic acid sequence of the light chain variable region: (378 bp)

CAGGCTGTTGTGACTCAGGAATCTGCACTCACCACATCACCTGGTGAAAC
AGTCACACTCACTTGTCGCTCAAGTACTGGGGCTGTTACAACTAGTAACTTTG
CCAACTGGGTCCAAGAAAAACCAGATCATTTATTCACTAGTCTAATAGGTGGTA
CCAACAACCGAGCTCCAGGTGTTCCTGCCAGATTCTCAGGCTCCCTGATTGGA
GACAAGGCTGCCCTCACCATCACAGGGGCACAGACTGAGGATGAGGCAATAT
ATTTCTGTGCTCTATGGTACAGCAACCATTGGGTGTTCGGTGGAGGAACCAAA
CTGACTGTCCTAGGCCAGCCCAAGTCTTCGCCATCAGTCACCCTGTTTCAAGG
GCAATTCTGC(SEQ ID NO: 3)

Encoded amino acid sequence: (126 aa)

QAVVTQESALTTSPGETVTLTCRSSTGAVTTSNFANWVQEKPDHLFTSLIGGT
NNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNHWVFGGGTKLTV
LGQPKSSPSVTLFQGQFC(SEQ ID NO: 4)

Example 3: Design and preparation of humanized antibodies 4G10H1L1, 4G10H3L3

and 4G10H4L3 against CTLA4

1. Design of light and heavy chain sequences of anti-CTLA4 humanized antibodies 4G10H1L1, 4G10H3L3 and 4G10H4L3

Based on the three-dimensional crystal structure of CTLA4 protein (Nat. Struct. Biol., (1997) 4 p.527) and the amino acid sequence of antibody 4G10 obtained in the Example 2, antibody *in silico* modeling was performed and mutations of amino acids from mouse-like to human-like were engineered to obtain the amino acid sequences of variable regions of antibody 4G10H1L1, 4G10H3L3 and 4G10H4L3 (the constant region of heavy chain was Ig gamma-1 chain C region, ACCESSION: P01857 and the constant region of light chain was Ig kappa chain C region, ACCESSION: P01834)

The designed sequences of variable regions are as follows

(1) The heavy chain and light chain sequences of the humanized monoclonal antibody 4G10H1L1

Nucleic acid sequence of the heavy chain variable region: (345 bp)

CAGGTGCAGCTGGTGGAGTCTGGGGCCGAGCTGGTGAAGCCCGGCGCCT
CCATGAAGATCTCTTGCAAGGCCAGCGGATACAGTTTCACTGGCTATACCATGA
ACTGGGTCAAACAGGCTCCAGGACAGGGACTGGAGTGGATCGGGCTGATTAA
TCCTTACAACAACATCACCAACTACAACCAGAAGTTCATGGGAAAAGCAACC
TTTACAGTGGACAAGAGCATTTCCACAGCCTACATGGAACTGAGCCGGCTGAC
TTCAGACGATAGCGGGGTCTATTTTTGTGCAAGGCTGGATTATCGCTCTTACTG
GGGGCAGGGAACTCTGGTCACTGTCTCCGCT(SEQ ID NO: 5)

Encoded amino acid sequence: (115 aa)

QVQLVESGAELVKPGASMKISCKASGYSFTGYTMNWVKQAPGQGLEWIGLI
NPYNNITNYNQKFMGKATFTVDKSISTAYMELSRLTSDDSGVYFCARLDYRSYW
GQGTLVTVSA(SEQ ID NO: 6)

Nucleic acid sequence of the light chain variable region: (327 bp)

CAGGCTGTCGTCACTCAGGAACCTTCACTGACTGTGAGCCCAGGAGGAA
CTGTCACCCTGACATGCGGAAGCTCCACCGGAGCAGTGACCACATCCAACTTC
GCCAATTGGGTCCAGGAAAAGCCAGGCCAGGCATTTCGATCCCTGATCGGAG
GCACAAACAATCGGGCTTCTTGGGTGCCCGCAAGATTCTCAGGAAGCCTGCT

GGGGGGAAAAGCCGCTCTGACCATTAGTGGCGCTCAGCCTGAGGACGAAGCC
GAGTACTTCTGCGCTCTGTGGTATAGCAACCACTGGGTGTTTGGCGGGGGAAC
AAAGCTGACTGTGCTG(SEQ ID NO: 7)

Encoded amino acid sequence: (109 aa)

QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNFANWVQEKPGQAFRSLIGGT
NNRASWVPARFSGSLLGGKAALTISGAQPEDEAEYFCALWYSNHWVFGGGTKLT
VL(SEQ ID NO: 8)

(2) The heavy chain and light chain sequences of the humanized monoclonal antibody 4G10H3L3

Nucleic acid sequence of the heavy chain variable region: (345 bp)

CAGGTGCAGCTGGTCGAGTCTGGGGCCGAAGTGAAGAAACCCGGCGCCT
CAGTGAAGGTCAGCTGCAAGGCCAGCGGGTACAGTTTCACTGGATATACCATG
AACTGGGTCCGACAGGCCCCTGGCCAGGGGCTGGAGTGGATCGGCCTGATTA
ACCCTTACAACAACATCACTAACTACGCACAGAAGTTCCAGGGGAGAGTGAC
CTTTACAGTGGACACCAGCATTTCCACAGCCTACATGGAACTGTCCCGGCTGA
GATCTGACGATACAGGCGTGTACTTCTGCGCTAGGCTGGATTACCGCAGCTATT
GGGGACAGGGCACACTGGTGACTGTCAGCGCA(SEQ ID NO: 9)

Encoded amino acid sequence: (115 aa)

QVQLVESGAEVKKPGASVKVSCKASGYSFTGYTMNWVRQAPGQGLEWIGL
INPYNNITNYAQKFQGRVTFTVDTSISTAYMELSRLRSDDTGVYFCARLDYRSYW
GQGTLVTVSA(SEQ ID NO: 10)

Nucleic acid sequence of the light chain variable region: (327 bp)

CAGGCTGTCGTCACTCAGGAACCTTCACTGACCGTGTCTCCTGGCGGGAC
TGTCACCCTGACATGCGGCAGCTCCACAGGGGCCGTGACCACAAGTAACTTC
CCAAATTGGGTCCAGCAGAAGCCAGGACAGGCTCCCCGGAGTCTGATCGGAG
GCACCAACAACAAGGCCAGCTGGACACCCGCACGGTTCAGCGGCAGCCTGCT
GGGCGGCAAGGCCGCTCTGACAATTAGCGGAGCCCAGCCTGAGGACGAAGCC
GAGTACTATTGCGCTCTGTGGTACTCCAACCACTGGGTGTTCGGCGGCGGCAC
CAAGCTGACTGTGCTG(SEQ ID NO: 11)

Encoded amino acid sequence: (109 aa)

QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNFPNWVQQKPGQAPRSLIGGT NNKASWTPARFSGSLLGGKAALTISGAQPEDEAEYYCALWYSNHWVFGGGTKLT VL(SEQ ID NO: 12)

(3) The heavy and light chain sequences of the humanized monoclonal antibody 4G10H4L3

Nucleic acid sequence of the heavy chain variable region: (345bp)

CAGGTGCAGCTGGTCGAGTCTGGGGCCGAAGTGAAGAAACCCGGCGCCT CAGTGAAGGTCAGCTGCAAGGCCAGCGGGTACAGTTTCACTGGATATACCATG AACTGGGTCCGACAGGCCCCTGGCCAGGGGCTGGAGTGGATCGGCCTGATTA ACCCTTACAACGACATCACTAACTACGCACAGAAGTTCCAGGGGAGAGTGAC CTTTACAGTGGACACCAGCATTTCCACAGCCTACATGGAACTGTCCCGGCTGA GATCTGACGATACAGGCGTGTACTTCTGCGCTAGGCTGGATTACCGCAGCTATT GGGGACAGGGCACACTGGTGACTGTCAGCGCA(SEQ ID NO: 13)

Encoded amino acid sequence: (115 aa)

QVQLVESGAEVKKPGASVKVSCKASGYSFTGYTMNWVRQAPGQGLEWIGL INPYNDITNYAQKFQGRVTFTVDTSISTAYMELSRLRSDDTGVYFCARLDYRSYW GQGTLVTVSA(SEQ ID NO: 14)

The nucleic acid and encoded amino acid sequences of the light chain variable region are the same as those of 4G10H3L3.

2. Preparation of humanized antibodies 4G10H1L1, 4G10H3L3 and 4G10H4L3

The constant region of heavy chain was Ig gamma-1 chain C region, ACCESSION: P01857. The constant region of light chain was Ig kappa chain C region, ACCESSION: P01834.

The heavy chain cDNAs and light chain cDNAs of 4G10H1L1, 4G10H3L3, 4G10H4L3 were separately cloned into pUC57simple vectors to obtain pUC57simple-4G10H1 and pUC57simple-4G10L1, pUC57simple-4G10H3 and pUC57simple-4G10L3, and pUC57simple-4G10H4 and pUC57simple-4G10L3, repectively. They were subcloned into pcDNA3.1 vectors. The recombinant plasmids were transfected into 293F cells and the culture medium was harvested and purified to

obtain humanized antibodies 4G10H1L1, 4G10H3L3 and 4G10H4L3. The purified 4G10H1L1 and 4G10H3L3 were identified by SDS-PAGE electrophoresis, and the result was shown in Figure 2 and Figure 3 respectively.

Example 4: Preparation of anti-PD-1 antibody 14C12

1. Establishment of hybridoma cell line LT003

Using PD-1-mFc as the antigen, the hybridoma cells were obtained by fusing the splenocytes of immunized BALB/C mice (purchased from Guangdong Medical Laboratory Animal Center) and mice myeloma cells with currently established method (for example, Stewart, S.J., "Monoclonal Antibody Production", in Basic Methods in antibody Production and Characterization, Eds.G.C. Howard and D.R. Bethell, Boca Raton: CRC Press, 2000).

Microplate was coated with PD-1-mFc as the antigen, and indirect ELISA was used to screen those hybridoma cells secreting new antibodies specifically binding to PD-1.

The hybridoma cells were further screened by competitive ELISA to select those secreting antibodies that competitively bind to PD-1 against ligand PDL1-hFc (PDL1 Genbank ID:NP_054862.1), and then a stable hybridoma cell line LT003(PD-1-14C12) was obtained by limited dilution method, and its secreted monoclonal antibody is named 14C12.

LT003 (PD-1-14C12), a hybridoma cell line, was deposited in China Center for Type Culture Collection (CCTCC) on June 16, 2015. Deposit Accession NO.: C2015105, depository address: Wuhan university, Wuhan, China, zip code: 430072.

2. Preparation of anti-PD-1 antibody 14C12

The LT003 cells in the present invention were cultured using IMDM medium containing 10% low IgG fetal bovine serum (IMDM medium containing 1% streptomycin, cultured in cell incubator with 5% CO2, 37°C incubator), and after 7 days culture the cell culture supernatant was harvested and purified to get the antibody 14C12.

Example 5: Acquisition of the sequence of antibody 14C12

Acquisition of the sequence of antibody 14C12

mRNA was extracted from the hybridoma cell line LT003 prepared in Example 4 above according to the manual of the cell/bacterial total RNA extraction reagent kit (Tiangen, Product No. DP430).

cDNA was synthesized using Invitrogen SuperScript ® III First - Strand Synthesis System for RT-PCR, and amplified by PCR.

TA cloning was directly carried out on the PCR amplified product according to the instructions of pEASY-T1 Cloning Kit (Transgen CT101).

The products of TA cloning were directly sequenced, and the sequencing results were as follows:

Nucleic acid sequence of heavy chain variable region: (354bp)

GAGGTCAAACTGGTGGAGAGCGGCGGCGGGCTGGTGAAGCCCGGCGGGT
CACTGAAACTGAGCTGCGCCGCTTCCGGCTTCGCCTTTAGCTCCTACGACATG
TCATGGGTGAGGCAGACCCCTGAGAAGCGCCTGGAATGGGTCGCTACTATCAG
CGGAGGCGGGCGATACACCTACTATCCTGACTCTGTCAAAGGGAGATTCACAA
TTAGTCGGGATAACGCCAGAAATACTCTGTATCTGCAGATGTCTAGTCTGCGGT
CCGAGGATACAGCTCTGTACTATTGTGCAAACCGGTACGGCGAAGCATGGTTT
GCCTATTGGGGACAGGGCACCCTGGTGACAGTCTCTGCC(SEQ ID NO: 15)

Encoded amino acid sequence: (118 aa)

EVKLVESGGGLVKPGGSLKLSCAASGFAFSSYDMSWVRQTPEKRLEWVATIS
GGGRYTYYPDSVKGRFTISRDNARNTLYLQMSSLRSEDTALYYCANRYGEAWFA
YWGQGTLVTVSA(SEQ ID NO: 16)

Nucleic acid sequence of the light chain variable region: (318 bp)

GACATTAAGATGACACAGTCCCCTTCCTCAATGTACGCTAGCCTGGGCGAG
CGAGTGACCTTCACATGCAAAGCATCCCAGGACATCAACACATACCTGTCTTG
GTTTCAGCAGAAGCCAGGCAAAAGCCCCAAGACCCTGATCTACCGGGCCAAT
AGACTGGTGGACGGGGTCCCCAGCAGATTCTCCGGATCTGGCAGTGGGCAGG
ATTACTCCCTGACCATCAGCTCCCTGGAGTATGAAGACATGGGCATCTACTATT
GCCTGCAGTATGATGAGTTCCCTCTGACCTTTGGAGCAGGCACAAAACTGGAA
CTG(SEQ ID NO: 17)

Encoded amino acid sequence: (106 aa)

DIKMTQSPSSMYASLGERVTFTCKASQDINTYLSWFQQKPGKSPKTLIYRAN
RLVDGVPSRFSGSGSGQDYSLTISSLEYEDMGIYYCLQYDEFPLTFGAGTKLEL(SE
Q ID NO: 18)

Example 6: Design, preparation and assay of humanized antibody 14C12H1L1 against PD-1

1. Design of the light and heavy chain sequences of the humanized antibody 14C12H1L1

Based on the three-dimensional crystal structure of PD-1 protein (Shinohara T, et al., Structure and chromosomal localization of the human PD-1 gene (PDCD1). Genomics 1995, 23 (3): 704–6) and the amino acids sequence of antibody 14C12 obtained in the Example 5, antibody *in silico* modeling was performed and mutations of amino acids from mouse-like to human-like were engineered to obtain the amino acid sequences of variable regions of antibody 14C12H1L1.

The designed sequences of variable regions are as follows:

Nucleic acid sequence of the heavy chain variable region: (354bp)

GAAGTGCAGCTGGTCGAGTCTGGGGGAGGGCTGGTGCAGCCCGGCGGGT
CACTGCGACTGAGCTGCGCAGCTTCCGGATTCGCCTTTAGCTCCTACGACATG
TCCTGGGTGCGACAGGCACCAGGAAAGGGACTGGATTGGGTCGCTACTATCTC
AGGAGGCGGGAGATACACCTACTATCCTGACAGCGTCAAGGGCCGGTTCACA
ATCTCTAGAGATAACAGTAAGAACAATCTGTATCTGCAGATGAACAGCCTGAG
GGCTGAGGACACCGCACTGTACTATTGTGCCAACCGCTACGGGGAAGCATGGT
TTGCCTATTGGGGGCAGGGAACCCTGGTGACAGTCTCTAGT (SEQ ID NO: 19)

Encoded amino acid sequence: (118 aa)

EVQLVESGGGLVQPGGSLRLSCAASGFAFSSYDMSWVRQAPGKGLDWVATIS
GGGRYTYYPDSVKGRFTISRDNSKNNLYLQMNSLRAEDTALYYCANRYGEAWFA
YWGQGTLVTVSS(SEQ ID NO: 20)

Nucleic acid sequence of the light chain variable region: (321 bp)

GACATTCAGATGACTCAGAGCCCCTCCTCCATGTCCGCCTCTGTGGGCGAC
AGGGTCACCTTCACATGCCGCGCTAGTCAGGATATCAACACCTACCTGAGCTG

GTTTCAGCAGAAGCCAGGGAAAAGCCCCAAGACACTGATCTACCGGGCTAAT
AGACTGGTGTCTGGAGTCCCAAGTCGGTTCAGTGGCTCAGGGAGCGGACAGG
ACTACACTCTGACCATCAGCTCCCTGCAGCCTGAGGACATGGCAACCTACTAT
TGCCTGCAGTATGATGAGTTCCCACTGACCTTTGGCGCCGGGACAAAACTGGA
GCTGAAG(SEQ ID NO: 21)

Encoded amino acid sequence: (107 aa)

DIQMTQSPSSMSASVGDRVTFTCRASQDINTYLSWFQQKPGKSPKTLIYRAN
RLVSGVPSRFSGSGSGQDYTLTISSLQPEDMATYYCLQYDEFPLTFGAGTKLELK(
SEQ ID NO: 22)

2. Preparation and SDS-PAGE electrophoresis of humanized antibody 14C12H1L1

The constant region of heavy chain is Ig gamma-1 chain C region, ACCESSION: P01857; and the constant region of light chain is Ig kappa chain C region, ACCESSION: P01834.

The heavy chain cDNA and light chain cDNA of 14C12H1L1 were separately cloned into pcDNA3.1 vector to obtain the recombinant expression plasmids. The recombinant plasmids were transfected into 293F cells. The 293F cell culture medium was purified and tested. As shown in Figure 4, the reduced target protein appeared at approximately 24.5 kD and 49 kD, while the non-reduced target protein appeared at approximately 147 kD.

Example 7: Sequence design, expression and assay of heavy chains and light chains of bispecific antibody BiAb001, BiAb002, BiAb003, BiAb004, BiAb007 and BiAb010

1. Sequence design

Bispecific antibody BiAb001, BiAb002, BiAb003, BiAb004, BiAb007 and BiAb010 in the present invention all belong to Morrison design (IgG-scFv), in which each heavy chain of an IgG antibody are connected with a scFv fragment of another antibody. The configurations of the heavy chains and light chains are shown in Table 1 below.

Table1. The configurations of BiAb001, BiAb002, BiAb003, BiAb004, BiAb007 and BiAb010 heavy chains and light chains

Bispecific Antibody	Heavy chains			Light chains
	IgG	Linker fragment	scFv	
BiAb001	14C12H1	Linker 1	4G10H1$_V$- Linker 2 -4G10L1$_V$	14C12L1
BiAb002	14C12H1	Linker 2	4G10H1$_V$- Linker 2-4G10L1$_V$	14C12L1
BiAb003	14C12H1	Linker 1	4G10H3$_V$- Linker 2 -4G10L3$_V$	14C12L1
BiAb004	14C12H1	Linker 2	4G10H3$_V$- Linker 2 -4G10L3$_V$	14C12L1
BiAb007	14C12H1	Linker 2	4G10H4$_V$- Linker 2 -4G10L3$_V$	14C12L1
BiAb010	14C12H1	Linker 2	8D2H14$_V$- Linker 2 -8D2L2$_V$	14C12L1

In Table 1:

(1) The antibody sequences marked with subscript "V" refer to the variable region of heavy chains or light chains. Those with no subscript "V" are full-length heavy chains or light chains with constant region. These variable regions or full-length sequences of amino acids and their coding nucleic acid sequences embody the corresponding sequences recorded in the examples above.

(2) Linker 1 amino acid sequence is (GGGGS)3 (SEQ ID NO: 23)

Linker 2 amino acid sequence is (GGGGS)4 (SEQ ID NO: 24)

(3) Amino acid sequence of the heavy chain variable region of 8D2H14L2 (8D2H14$_V$):

EVQLVESGGGLVQPGGSSRLSCAASGFTFSDNWMNWVRQAPGKGLEWLAQ IRNKPYNYETYYSASVKGRFTISRDDSKNSVYLQMNSLKTEDTGVYYCTAQFAY WGQGTLVTVSS(SEQ ID NO: 25)

Encoded nucleic acid sequence of 8D2H14$_V$:

GAGGTGCAGCTGGTCGAATCTGGAGGAGGACTGGTGCAGCCTGGAGGAA GCTCCCGGCTGTCATGTGCCGCTAGCGGCTTCACCTTTTCCGACAACTGGATG AATTGGGTGCGACAGGCACCAGGCAAAGGACTGGAGTGGCTGGCTCAGATCC GGAACAAGCCCTACAATTATGAAACATACTATAGCGCCTCCGTGAAAGGCCGG TTCACTATTAGTAGAGACGATTCTAAGAACAGCGTGTACCTGCAGATGAATAGC CTGAAGACAGAGGATACTGGCGTCTACTATTGCACAGCACAGTTTGCCTATTG GGGACAGGGCACCCTGGTGACAGTCTCTAGT(SEQ ID NO: 26)

(4) Amino acid sequence of the light chain variable region of 8D2H14L2 ($8D2L2_V$) :

DIQMTQSPSSLSASVGDRVTITCRTSENIYGGLNWYQRKPGKSPKLLIYGATN LASGVSSRFSGSGSGTDYTLTISSLQPEDVATYYCQNVLRSPFTFGSGTKLEIK(SE Q ID NO: 27)

Encoded nucleic acid sequence of $8D2L2_V$:

GACATCCAGATGACTCAGAGCCCCTCAAGCCTGTCTGCAAGTGTGGGCGA TAGGGTCACCATCACATGTCGCACCTCCGAAAACATCTACGGGGGACTGAATT GGTATCAGCGCAAGCCCGGCAAATCCCCTAAGCTGCTGATCTACGGCGCTACC AACCTGGCATCTGGGGTGTCCTCTCGATTTTCAGGGAGCGGCAGCGGCACCG ACTATACTCTGACCATTAGTTCACTGCAGCCTGAGGATGTGGCCACATACTATT GCCAGAATGTCCTGAGATCACCATTCACTTTTGGGAGCGGAACCAAACTGGA AATTAAG(SEQ ID NO: 28)

2. Expression and purification of antibody BiAb001

cDNAs of heavy chain and light chain of BiAb001 were separately cloned into pUC57simple vectors (provided by GenScript) to obtain plasmids pUC57simple-BiAb001H and pUC57simple- BiAb001L, respectively.

pUC57simple-BiAb001H and pUC57simple-BiAb001L were individually digested with enzymes (HindIII&EcoRI), and genes of heavy chain and light chain recovered via electrophoresis were sub-cloned into pcDNA3.1 vector, respectively. The recombinant plasmids were extracted and co-transfected into 293F cells. After 7 days culture, the culture supernatant was harvested by high-speed centrifugation and concentration, and purified by loading onto HiTrap protein A HP column and eluting with Elution Buffer in one step to obtain the antibody and stored in PBS.

The purified antibody samples were added to reduced protein electrophoresis loading buffer and non-reduced protein electrophoresis loading buffer, respectively. After being boiled, the samples were examined on SDS-PAGE electrophoresis. The results of BiAb001 electrophoresis was shown in Figure 5, in which the reduced protein sample appeared at 23.6 kD and 75.8 kD, and the non-reduced protein sample (individual antibody) appeared at 199 kD.

3. Expression and purification of antibody BiAb002, BiAb003, BiAb004, BiAb007

and BiAb010

Purified antibodies of BiAb002, BiAb003, BiAb004, BiAb007 and BiAb010 were obtained according to the aforementioned methods used for preparation of BiAb001.

The purified antibodies samples were added into of reduced protein electrophoresis loading buffer and non-reduced protein electrophoresis loading buffer, respectively. After being boiled, the samples were examined on SDS-PAGE electrophoresis. The results of BiAb002, BiAb003, BiAb004, BiAb007 and BiAb010 electrophoresis were shown in Figure 6, 7, 8, 9 and 10, respectively, in which the reduced protein sample appeared at 23.6 kD and 75.8 kD, and the non-reduced protein sample (individual antibody) appeared at 199 kD.

Example 8: Determination of antibody binding kinetics

The binding kinetics of antigen and antibody were measured by Fortebio molecular interaction instrument.

1. Binding kinetics of antibody 4G10 and its humanized antibody 4G10H1L1, 4G10H3L3, and 4G10H4L3 to antigen CTLA4 were measured

1.1 CTLA4 antigen was obtained by digesting CTLA4-mFc with TEV protease and column purification

1.2 Antibody 4G10 was immobilized to AR2G Biosensors by amine coupling method, and then blocked with ethanolamine and equilibrating in PBST, and then bound to CTLA4. CTLA4 was double gradient diluted with PBST to the concentrations of 268.1, 134.1, 67, 33.5, 16.8, 8.38, 4.19, and 0 nM. The dissociation was also in PBST. Humanized antibodies 4G10H1L1, 4G10H3L3and 4G10H4L3 were measured with similar methods to that of 4G10, with antigen concentrations of 180, 90, 45, 22.5, 11.25, 5.625, 2.813 and 0nM.

1.3 The binding kinetics of antibody 4G10 and its humanized antibodies 4G10H1L1, 4G10H3L3, and 4G10H4L3 to antigen CTLA4 are shown in Table 1 below, and in Figure 11, Figure 12, Figure 13 and Figure 14, respectively.

2. Binding kinetics of antibody 14C12 and its humanized antibody 14C12H1L1 to

antigen PD-1

2.1 PD-1 antigen was obtained by digesting PD-1-mFc with TEV protease and column purification

2.2 The antigen PD-1 (antigen concentration of 1 μg/ml) was immobilized on the surface of SA sensor after being labeled with biotin, and after equilibrating in PBST it bind to antibodies 14C12 and 14C12H1L1, respectively. The antibodies were diluted with PBST from 200 nM down three fold each time, and the dissociation was also in PBST.

2.3 The binding kinetics of antibodies 14C12 and 14C12H1L1 to antigen are shown in Table 1 below and in Figures 15 and 16.

3. Binding kinetics of antibodies BiAb001, BiAb002, BiAb003, BiAb004, BiAb007 and BiAb010 to antigen CTLA4.

3.1 CTLA4 (antigen concentration of 1 μg/ml) was immobilized on the surface of SA sensor after being labeled with biotin, and after equilibrating in PBST, it binds to antibodies BiAb001, BiAb002, BiAb003, BiAb004, BiAb007 and BiAb010, respectively. The antibodies were diluted with PBST from 200 nM down three fold each time. The dissociation was also in PBST.

3.2 Binding kinetics of antibodies BiAb001, BiAb002, BiAb003, BiAb004, BiAb007 and BiAb010 to antigen CTLA4 are shown in Table 1 and in Figures 17-21, respectively.

4. Binding kinetics of antibodies BiAb001, BiAb002, BiAb003, BiAb004, BiAb007 and BiAb010 to antigen to antigen PD-1

4.1 The antigen PD-1 (antigen concentration of 1 μg/ml) was immobilized on the surface of SA sensor after being labeled with biotin, and after equilibrating in PBST, it binds to antibodies BiAb001, BiAb002, BiAb003, BiAb004, BiAb007 and BiAb010, respectively. The antibodies were diluted with PBST from 200 nM down three fold each time. The dissociation was also in PBST.

4.2 Binding kinetics of antibodies BiAb001, BiAb002, BiAb003, BiAb004, BiAb007 and BiAb010 to antigen PD-1 are shown in Table 2, and in Figures 22- Figures 27, respectively.

Table 2：Kinetic parameters of antibody binding to antigen

Antibody	Antigen	K_D (M)	Kon(1/Ms)	Kon Error	Kdis(1/s)	Kdis Error
4G10	CTLA4 1 μg/ml	3.01E-10	3.78E+05	4.36E+03	1.14E-04	5.33E-06
4G10 H1L1		1.52E-09	1.86E+05	3.26E+03	2.82E-04	9.23E-06
4G10 H3L3		4.14E-09	2.09E+05	3.81E+03	8.64E-04	1.11E-05
4G10H4L3		9.67E-10	1.37E+05	2.22E+03	1.32E-04	8.69E-06
14C12	PD-1 1 μg/ml	1.81E-11	3.38E+05	8.23E+03	6.12E-06	1.04E-05
14C12H1L1		2.42E-11	3.17E+05	5.90E+03	7.66E-06	8.70E-06
BIAb001	CTLA4 1 μg/ml	1.67E-10	2.33E+05	4.45E+03	3.89E-05	8.75E-06
BIAb002		9.69E-11	2.37E+05	5.32E+03	2.30E-05	9.97E-06
BIAb003		3.95E-10	3.60E+05	7.10E+03	1.42E-04	9.99E-06
BIAb004		5.66E-10	2.20E+05	3.89E+03	1.24E-04	8.27E-06
BiAb007		2.72E-10	1.58E+06	5.17E+04	4.28E-04	1.12E-05
BiAb010		3.22E-10	1.08E+06	1.99E+04	3.47E-04	7.28E-06
BIAb001	PD-1 1 μg/ml	4.16E-11	2.97E+05	4.96E+03	1.24E-05	8.36E-06
BIAb002		3.33E-11	2.20E+05	5.93E+03	7.32E-06	1.15E-05
BIAb003		4.12E-11	2.64E+05	5.49E+03	1.09E-05	9.82E-06
BIAb004		4.82E-11	2.47E+05	5.45E+03	1.19E-05	9.61E-06
BiAb007		1.40E-11	4.52E+05	9.23E+03	6.30E-06	7.85E-06
BiAb010		2.97E-11	2.28E+05	4.40E+03	6.79E-06	8.70E-06

K_D Is the affinity constant; K_{on} is the association rate of antigen-antibody. K_{dis} is the dissociation rate of antigen-antibody; $K_D = K_{dis}/K_{on}$.

The results showed that:

the antibody 4G10 and its humanized antibodies have good affinity to the antigen CTLA4. Both antibodies 14C12 and 14C12H1L1 have good affinity to antigen PD-1.

Bispecific antibodies have good affinity to antigen CTLA4 and PD-1.

Example 9: The binding activity of antibody to antigen measured by ELISA

1. The binding activity of humanized antibodies 4G10H1L1 and 4G10H3L3 to antigen CTLA4

1.1 The binding activity of humanized antibodies 4G10H1L1 and 4G10H3L3 to CTLA4 was determined by indirect ELISA.

After incubated with antigen at 4°C overnight, the microplate was blocked with 1% BSA at 37°C for 2h, and then the antibodies were added and incubated at 37°C for 30 min, and then HRP-labeled secondary antibody (goat anti-human IgG (H+L)) (Jackson, 109-035-088) was added and incubated at 37°C for 30 min. TMB (Neogen, 308177) was added to react for 5 mins. The absorbance was read at the wavelength of 450 nm in a microplate reader.

The binding results were shown in Figure 28. As shown in the fingure, both 4G10H1L1 and 4G10H3L3 can bind to CTLA4 protein effectively with dose-dependency. The absorbance intensities at different doses were shown in Table 3. Through Curve Simulation using quantitative analyses of absorbance values, EC50 of 4G10H1L1 and 4G10H3L3 were then determined to be 0.048 nM and 0.067 nM, respectively.

Table 3 : The binding activity of 4G10H1L1 and 4G10H3L3 to CTLA4 was measured by indirect ELISA

Coating Antigen: CTLA4 0.5 μg/ml				
Serial dilution of antibody	4G10 H1L1		4G10 H3L3	
6 μg/ml	2.926	2.946	2.809	2.764
1:5	2.784	2.732	2.729	2.739
1:25	2.729	2.688	2.668	2.617
1:125	2.490	2.469	2.367	2.309
1:625	1.736	1.709	1.498	1.357
1:3126	0.607	0.663	0.513	0.432
1:16525	0.198	0.225	0.175	0.149
1:78125	0.096	0.115	0.089	0.087
1:390625	0.075	0.087	0.075	0.072

1:1953125	0.071	0.090	0.066	0.077
1:9765625	0.066	0.087	0.078	0.089
0	0.073	0.079	0.079	0.068
Secondary antibody	Goat anti-Human IgG, HRP(1:5000)			

1.2. The binding activity of humanized antibodies 4G10H1L1 and 4G10H3L3 to CTLA4 by competition ELISA against B7

Coating antigen to microplate with B7/1-hFc (B7/1 genbank ID: NP 005182.1) 4 ℃ overnight, and then after blocked with 1% BSA for 2 hours, mixtures of antibodies and CTLA4-mFc antibody were added (dilute concentrations are shown in table 4) and incubate for 30 min at 37 ℃; and then secondary antibody labeled with enzyme was added and then incubated for 30 mins at 37 ℃. The absorption value of 450 nm was measured on the enzyme-labeled instrument (see table 4).

The binding results of antibodies to CTLA4 competing against B7-1 were shown in Figure 29. As showen in the figure, the antibodies 4G10H1L1 and 4G10H3L3 can compete against B7-1 and bind to CTLA4 protein effectively with dose-dependency. The absorbance at different doses were shown in Table 4. Through Curve Simulation using quantitative analyses of absorbance values, EC50 of 4G10H1L1 and 4G10H3L3 binding with CTLA4 were then determined to be 1.297nm and 1.229nm, respectively.

Table 4 : The binding activity of humanized antibodies 4G10H1L1 and 4G10H3L3 to CTLA4 by competition ELISA against B7

Coating Antigen:B7/1-hFc 0.5 μg/ml					
Serial dilution of Antibody	4G10H1L1		4G10H3L3		receptor
3 μg/ml	0.132	0.121	0.146	0.185	CTLA4-mFc

1:3	0.120	0.170	0.159	0.182	0.3μg/ml
1:9	0.260	0.343	0.382	0.340	
1:27	0.399	0.593	0.570	0.507	
1:81	0.565	0.614	0.642	0.642	
1:243	0.628	0.753	0.784	0.773	
1:729	0.573	0.760	0.768	0.702	
1:2187	0.553	0.824	0.741	0.788	
1:6561	0.661	0.844	0.824	0.679	
1:19683	0.555	0.834	0.742	0.699	
1:59049	0.552	0.725	0.773	0.770	
0	0.610	0.665	0.822	0.717	
Secondary antibody	Goat anti-Mouse IgG, HRP (1:5000)				

2. The binding activities of monoclonal antibody 14C12 and its humanized antibody 14C12H1L1 to antigen PD-1 measured by ELISA

2.1 The binding activity of monoclonal antibodies 14C12 and 14C12H1L1 to antigen PD-1 was determined by indirect ELISA as follows:

After incubated with PD-1-mFc at 4°C overnight, the microplate was blocked with 1% BSA at 37°C for 2h, and the antibodies were added, incubated at 37°C for 30 min, and HRP-labeled secondary antibody (goat anti-human IgG (H+L)) (Jackson, 109-035-088) was added and incubated at 37°C for 30 min. TMB (Neogen, 308177) was added to react for 5 mins. The absorbance was read at the wavelength of 450 nm in a microplate reader.

The binding results of antibodies 14C12 and 14C12H1L1 to PD-1 were shown in Figure 30. Evidently, both 14C12 and 14C12H1L1 can bind to PD-1 protein effectively with dose-dependency. The absorbance at different doses were shown in Table 5. Through Curve Simulation using quantitative analyses of absorbance values, EC50 of 14C12 and 14C12H1L1 binding to PD-1 were then determined to be 0.175 nM and 0.043 nM, respectively.

Table 5：The binding activities of antibodies 14C12 and 14C12H1L1 to PD-1, respectively

Coating Antigen: PD-1-mFc (0.5μg/mL)				
Antibody concentration(μg/mL)	14C12		14C12H1L1	
1	2.463	2.439	2.643	2.557
0.3	2.572	2.380	2.734	2.586
0.1	2.118	2.126	2.633	2.535
0.03	1.607	1.438	2.384	2.335
0.01	0.930	0.809	1.892	1.839
0.003	0.407	0.346	1.115	1.011
0.001	0.167	0.150	0.503	0.455
0	0.062	0.047	0.068	0.064
Secondary antibody	Goat anti- Mouse secondary antibody, HRP Conjugate			

2.2. The binding activity of monoclonal antibody 14C12 produced by hybridoma and its humanized antibody 14C12H1L1 to antigen PD-1 by competition ELISA against PDL1 was measured as follows:

After incubated with PD-1-hFc or PD-1-mFc at 4°C overnight, the microplate was blocked with 1% BSA at 37°C for 2h; and then mixtures of individual antibody, 14C12 or 14C12H1L1, at different concentrations (see Table 6 for dilution gradient) and PDL1-hFc or PDL-1-mFc were added into the microplate to react for 10 mins; and then HRP-labeled secondary antibody was added and incubated at 37°C for 30 min. The absorbance was read at the wavelength of 450 nm in a microplate reader (see Table 6).

The binding results of antibodies to PD-1 competing against PDL1 were shown in Figure 31. the antibody 14C12 and its humanized antibody 14C12H1L1 can compete against PDL1 to bind to PD-1 protein effectively with dose-dependency. The absorbance intensities at different doses were shown in Table 6. By using quantitative analyses of absorbance values, EC50 of 14C12 and 14C12H1L1 binding with PD-1 that were calculated via Curve Simulation were then determined to be 0.853 nM and 0.37 nM, respectively.

Table 6： The binding activity of 14C12 and 14C12H1L1 to PD-1 by competition

ELISA against PDL1

Antibody concentration(μg/mL)	Coating antigen:PD-1-mFc 0.2μg/mL			
	14C12		14C12H1L1	
1.5μg/ml	0.111	0.088	0.135	0.113
1:3	0.100	0.116	0.130	0.131
1:9	0.645	0.643	0.260	0.185
1:27	1.463	1.614	0.257	0.218
1:81	1.841	1.686	0.355	0.350
1:243	1.983	1.769	0.399	0.364
1:729	1.789	1.770	0.417	0.411
0	1.791	1.790	0.430	0.402
PDL1-hFc 2μg/ml				
Secondary antibody	Goat anti- Mouse secondary antibody HRP Conjugate			

3. The binding activity of antibodies BiAb001、BiAb002、 BiAb003 and BiAb004 to antigens measured by ELISA

3.1 The binding activity of antibodies BiAb001, BiAb002, BiAb003, and BiAb004 to antigen CTLA-4 was determined by indirect ELISA (Refer to methods described in 1.1 of the present Example)

The binding results of antibodies BiAb001, BiAb002, BiAb003 and BiAb004 to antigen CTLA4 were shown in Figure 32. Evidently, antibodies BiAb001, BiAb002, BiAb003 and BiAb004 can bind to CTLA4 protein effectively with dose-dependency. The absorbance at different doses were shown in Table 7. Through Curve Simulation using quantitative analyses of absorbance values, EC50 of antibodies BiAb001, BiAb002, BiAb003 and BiAb004 binding to CTLA4 were then determined as shown in Table 7 below.

Table 7：The binding activity of bispecific antibodies BiAb001, BiAb002, BiAb003, and BiAb004 to antigen CTLA4 (Indirect ELISA)

Serial	Coating Antigen:CTLA4 0.5 μg/ml

dilution of Antibody	BiAb001		BiAb002		BiAb003		BiAb004	
6μg/ml	2.425	2.098	2.334	2.120	2.179	2.076	2.243	2.251
1:3	2.299	2.234	2.204	2.257	2.141	2.138	2.198	2.319
1:9	2.265	2.188	2.168	2.186	2.012	2.086	2.207	2.254
1:27	2.245	2.215	2.174	2.043	1.814	1.811	1.982	1.907
1:81	1.859	1.856	1.717	1.609	1.438	1.410	1.534	1.640
1:243	1.494	1.511	1.221	1.136	0.933	0.899	1.070	1.108
1:729	0.818	0.922	0.644	0.610	0.451	0.414	0.567	0.548
0	0.048	0.048	0.048	0.047	0.047	0.045	0.049	0.050
Secondary antibody: Goat anti- human IgG，HRP(1:5000)								
EC50 (nM)	0.105		0.12		0.189		0.154	

3.2 The binding activity of antibodies BiAb001, BiAb002, BiAb003, and BiAb004 to antigen PD-1 was determined by indirect ELISA. (Refer to methods described in 2.1 of the present Example)

The binding results of antibodies BiAb001, BiAb002, BiAb003, and BiAb004 to antigen PD-1 were shown in Figure 33. Evidently, antibodies BiAb001, BiAb002, BiAb003, and BiAb004 can bind to PD-1 protein effectively with dose-dependency. The absorbance intensities at different doses were shown in Table 7. Through Curve Simulation using quantitative analyses of absorbance values, EC50 of antibodies BiAb001, BiAb002, BiAb003 and BiAb004 binding to PD-1 were then determined as shown in Table 8 below.

Table 8：The binding activity of bispecific antibodies to antigen CTLA4 (Indirect ELISA)

Serial dilution of Antibody	Coating Antigen:PD-1-mFc 0.5 μg/ml							
	BiAb001		BiAb002		BiAb003		BiAb004	
6μg/ml	2.400	2.360	2.370	2.314	2.332	2.290	2.347	2.343
1:3	2.450	2.426	2.290	2.388	2.271	2.326	2.410	2.458

1:9	2.402	2.457	2.372	2.346	2.279	2.351	2.390	2.505
1:27	2.409	2.467	2.332	2.348	2.350	2.243	2.414	2.396
1:81	2.375	2.254	2.084	1.990	1.996	1.928	2.197	2.175
1:243	1.871	1.725	1.627	1.544	1.414	1.419	1.573	1.560
1:729	1.067	1.047	0.954	0.814	0.746	0.719	0.920	0.865
0	0.085	0.067	0.065	0.068	0.055	0.055	0.056	0.058
Secondary antibody: Goat anti- human IgG, HRP (1:5000)								

3.3 The binding activity of the antibodies BiAb001, BiAb002, BiAb003, and BiAb004 to CTLA4 respectively by competition ELISA against B7/1-hFc (Refer to methods described in 1.2 of the present Example).

The binding results were shown in Figure 34. As showen in the figure, the antibodies BiAb001, BiAb002, BiAb003, and BiAb004 can effectively bind antigen CTLA4 and inhibit CTLA4 binding to B7/1 with dose-dependency. The absorbance intensities at different doses were shown in Table 9. Through Curve Simulation using quantitative analyses of absorbance values, EC50 of antibodies BiAb001, BiAb002, BiAb003 and BiAb004 were then determined as shown in Table 9 below.

Table 9：The binding activity of antibodies to CTLA4 by competition ELISA against B7/1-hFc

Serial dilution of Antibody	Coating Antigen:B7/1-hFc 0.5 μg/ml							
	BiAb001		BiAb002		BiAb003		BiAb004	
3μg/ml	0.076	0.072	0.078	0.095	0.074	0.080	0.095	0.076
1:3	0.081	0.076	0.079	0.079	0.095	0.086	0.097	0.100
1:9	0.748	0.706	1.040	1.031	1.029	1.049	0.907	0.973
1:27	1.153	1.129	1.076	1.152	1.125	1.361	1.010	1.056
1:81	1.121	1.241	1.153	1.315	1.241	1.198	1.121	1.206
1:243	1.261	1.236	1.047	1.266	1.333	1.335	1.231	1.235
1:729	1.063	1.077	1.085	1.337	1.210	1.323	1.157	1.287

0	1.0476	0.9808	0.9131	1.0762	1.067	1.074	1.032	0.966
Receptor： CTLA4-mFc 0.3μg/ml								
Secondary antibody：Goat anti- Mouse IgG，HRP Conjugate (1:5000)								
EC50 (nM)	2.758		1.797		2.197		2.256	

3.4 The binding activity of antibodies BiAb001, BiAb002, BiAb003, and BiAb004 to antigen PD-1 by competition ELISA against PDL1 (Refer to methods described in 2.2 of the present Example)

The binding results were shown in Figure 35. Evidently, the antibodies BiAb001, BiAb002, BiAb003, and BiAb004 can effectively bind antigen PD-1 and inhibit PD-1 binding to PDL1 with dose-dependency. The absorbance intensities at different doses were shown in Table 10. Through Curve Simulation using quantitative analyses of absorbance values, EC50 of antibodies BiAb001, BiAb002, BiAb003 and BiAb004 to CTLA4 were then determined as shown in Table 10 below.

Table 10：The binding activity of antibodies to PD-1 by competition ELISA against PDL1.

Antibody concentration	Coating antigen:PD-1-hFc 0.5 μg/ml							
	BiAb001		BiAb002		BiAb003		BiAb004	
3μg/ml	0.347	0.348	0.369	0.353	0.074	0.075	0.078	0.075
1:3	0.314	0.326	0.348	0.350	0.071	0.081	0.073	0.074
1:9	0.332	0.330	0.340	0.340	0.095	0.095	0.093	0.095
1:27	0.542	0.775	0.758	0.733	0.695	0.737	0.639	0.643
1:81	1.041	1.009	1.018	1.063	0.983	1.010	0.954	1.019
1:243	1.131	1.117	1.149	1.186	1.070	1.165	1.009	1.082
1:729	1.186	1.129	1.072	1.199	1.093	1.029	1.032	1.080
0	1.2345	1.1091	1.1243	1.1759	1.101	1.140	1.178	1.153
Receptor： PDL1-mFc 0.3μg/ml								
Secondary antibody：Goat anti- human IgG，HRP (1:5000)								
EC_{50} (nM)	0.685		0.543		0.665		0.62	

Example 10: The binding activity of antibodies to cell surface antigen by flow cytometry method

Host cells 293T expressing CTLA4 or PD-1 antigens were constructed respectively, and labeled with the humanized antibodies prepared in the present invention. The ability of the antibodies to bind specifically to corresponding cell surface antigens in its native conformation was analyzed and validated by flow cytometry.

1. Construction of 293T Host Cell Expressing CTLA4 or PD-1

293T cells were transfected with the CTLA4–containing plasmid pLenti6.3-CTLA4 or PD-1-containing plasmid pLenti6.3-PD-1 (vector pLenti6.3 was purchased from Invitrogen Corporation) and screened to obtain the stable pools of 293T-CTLA4 or 293T-PD-1 expressing CTLA4 or PD-1, respectively.

2. Antibody binding to cell surface antigens

The host cells obtained above that express individual antigen were digested by using trypsin, and distributed into tubes each containing 2×10^5 cells. Antibodies were diluted in gradient using PBSA buffer (1% BSA) and incubated with 293T cells that express corresponding antigens on ice for 2h. 100 μL of FITC-labeled goat anti-human IgG (1:500) was added into each tube and incubated on ice for 1h. After being washed with PBS for 3 times, cells were re-suspended in 300 μL of PBS, and fluorescence signals were measured on the flow cytometer using the FITC channel.

2.1 Binding activity of antibodies to cell surface antigens

The binding results of humanized antibodies 4G10H1L1 and 4G10H3L3 to 293T-CTLA4 cells were shown in Figure 36 and Figure 37. As shown in the figure, the antibodies 4G10H1L1 and 4G10H3L3 can effectively bind to target protein CTLA4 expressed on the surface of host cells 293T-CTLA4 with dose-dependency. The fluorescence intensities at different doses were shown in Table 11. Through Curve Simulation using quantitative analyses of absorbance values, EC_{50} of 4G10H1L1 and 4G10H3L3 binding to CTLA4 were determined to be 7.58 nM and 10.54 nM, respectively.

Table 11. The fluorescence intensities of antibodies 4G10H1L1 and 4G10H3L3 binding to CTLA4 expressed on 293T-CTLA4 cell surface by Flow Cytometry

	4G10H1L1	4G10H2L2
Antibody concentration (nM)	fluorescence intensity	
0.01	14.93	15.13
0.1	24.79	47.05
1	106.77	97.27
2.5	272.24	236.66
5	547.76	465.54
10	1080.91	788
20	1568.19	1296.95
50	1652.26	1539.24

2.2 The binding results of humanized antibody 14C12H1L1 to 293T-PD-1 cells were shown in Figure 38. As shown in the figure, the antibody 14C12H1L1 can effectively bind to target protein PD-1 expressed on the surface of host cells 293T-PD-1 with dose-dependency. The fluorescence intensities at different doses were shown in Table 12. Through Curve Simulation using quantitative analyses of fluorescence intensity, EC_{50} of 14C12H1L1 binding to PD-1 was determined to be 1.89 nM.

Table 12. The fluorescence intensity of antibody 14C12H1L1 binding to PD-1 expressed on 293T-PD-1 cell surface by Flow Cytometry.

Antibody concentration (nM)	0.01	0.1	1	5	10	50
fluorescence intensity	8.32	20.31	174.62	579.41	686.49	669.54

2.3 The binding results of the antibodies BiAb001, BiAb002, BiAb003, and BiAb004 to 293T-CTLA4 cells were shown in Figures 39-42. As shown in the figures, the antibodies BiAb001, BiAb002, BiAb003, and BiAb004 can effectively bind to target protein CTLA4 expressed on the surface of host cell 293T-CTLA4 with dose-dependency. The fluorescence intensities at different doses were shown in Table 13. Through Curve

Simulation using quantitative analyses of fluorescence intensities, EC_{50} of BiAb001, BiAb002, BiAb003, and BiAb004 were determined as shown in Table 13 below.

Table 13. The fluorescence intensities and EC_{50} of the antibodies BiAb001, BiAb002, BiAb003, and BiAb004 binding to CTLA4 expressed on 293T-CTLA4 cell surface by Flow Cytometry

Antibody concentration (nM)	BIAb001	BIAb002	BIAb003	BIAb004
	MFI (fluorescence intensity)			
0.0	-	-	12.9	13.0
0.1	10.5	10.5	14.2	15.6
1.0	21.2	20.4	28.7	34.8
2.5	46.8	43.8	49.6	77.4
5.0	92.9	93.5	101.0	129.6
10.0	181.9	171.2	245.3	313.0
20.0	312.8	282.1	487.4	608.6
50.0	469.5	466.5	899.8	1260.8
100.0	423.0	435.3	937.5	1020.6
200.0	381.6	408.2	-	-
EC50(nM)	11.9	13.7	19.9	17.8

2.4 The binding results of the antibodies BiAb001, BiAb002, BiAb003, and BiAb004 to 293T-PD-1 cells are shown in Figures 43-46. Evidently, the antibodies BiAb001, BiAb002, BiAb003, and BiAb004 can effectively bind to PD-1 expressed on the surface of host cell 293T-PD-1 with dose-dependency. The fluorescence intensities at different doses were shown in Table 14. Through Curve Simulation using quantitative analyses of absorbance values, EC_{50} of BiAb001, BiAb002, BiAb003, and BiAb004 were determined as shown in Table 14 below.

Table 14 : The fluorescence intensities of the antibodies BiAb001, BiAb002,

BiAb003, and BiAb004 binding to PD-1 expressed on 293T-PD-1 cell surface by Flow Cytometry

	BIAb001	BIAb002		BIAb003	BIAb004
Antibody concentration/nM	MFI (fluorescence intensity)		Antibody concentration/nM	MFI (fluorescence intensity)	
0.01	10.18	11	0.01	8.56	8.89
0.08	13.92	14.09	0.1	14.57	13.46
0.75	36.44	36.11	1	70.76	50.8
1.88	74.27	70.97	2.5	143.24	100.83
3.75	148.28	142.37	5	332.5	204.83
7.5	223.29	204.22	10	632.57	378.31
15	337.03	302.05	20	1026.03	535.69
37.5	358.78	290.92	50	958.92	734.73
			100	943.77	682.25
EC50(nM)	5.69	4.61		7.18	10

3. The binding activity of antibodies to T cell surface antigens CTLA4 and PD-1

PBMC was isolated by Ficoll-Paque Plus (GE Healthcare LOT No.:171440-02), and further isolated to get CD4+ cells, and then cells were stimulated with PHA for three days and then cells were washed once with PBS and mixed with antibodies at different concentrations, and then incubated on ice for 1.5 h. The cells were then washed with PBS once after incubation, and the FITC-labeled anti-human IgG (Jackson immunoresearch lot. 102155). Then the cells were incubated on ice in the dark for 1h, washed with PBS for once, and then the fluorescence signals were measured on the flow cytometer.

The control anti-PD-1 antibody Nivolumab is commercially available, and its information could also be found in http://www.drugbank.ca/drugs/DB09035;

The control anti-CTLA4 antibody Ipilimumab is commercially available, and its information can be found in http://www.drugbank.ca/drugs/DB06186.

3.1 The binding results of humanized antibody 4G10H3L3 to T cells were shown in Figure 47. As shown in figure, the antibody 4G10H3L3 can effectively bind to the target protein CTLA4 expressed on the surface of T cells with dose-dependency.

3.2 The binding results of humanized antibody 14C12H1L1 to T cells were shown in Figure 48. As shown in figure, the antibody 14C12H1L1 can effectively bind to the target PD-1 expressed on the surface of T cells with dose-dependency.

3.3 The binding activity to T cells of the antibodies BiAb003 and BiAb004 compared with that of 14C12H1L1 and 4G10H3L3 were shown in Figure 49. As shown in figure, the antibodies BiAb003, BiAb004, 14C12H1L1, and 4G10H3L3 can effectively bind to the target protein PD-1 expressed on the surface of T cells with dose-dependency. Furthermore, the binding activity of the antibodies BiAb003, BiAb004, and 14C12H1L1 to T cells were stronger than those of the antibodies 4G10H3L3, Nivolumab, and Ipilimumab. The fluorescence intensity was shown in Table 15.

Table 15：The fluorescence intensities of the antibodies 14C12H1L1, 4G10H3L3, BiAb003, and BiAb004 binding to T cells

Antibody concentration/nM	100	10	1
Name of antibody	MFI (fluorescence intensity)		
PBS	8.39	-	-
hIgG	10.15	-	-
Nivolumab	22.88	-	-
Ipilimumab	8.35	-	-
14C12H1L1	48.94	29.93	19.97
4G10H3L3	14.11	9.78	8.62
BIAb003	49.09	23.67	14.65
BIAb004	47.54	22.85	14.66

Example 11: Mixed lymphocyte reaction: secretion of cytokine IFN-γ and IL-2

PBMC was isolated by Ficoll-Paque Plus (GE Healthcare LOT No.: 171440-02), then mixed with IL-4 (Peprotech K2513, 1000 U/ml) and GM-CSF (Peprotech H1513, 1000 U/ml) to induce for 6 days, and then TNF-α (Peprotech G1513, 200 U/ml) was added to induce for 3 days to obtain DC cells.

T cells were isolated from PBMC and mixed with the DC cells ousedbtained above in the ratio of 1:10 to culture together with each antibody (hIgG wa as control) in different ratios for 5-6 days. The secretions of IFN-γ or IL-2 were measured with ELISA reagent kits (both purchased from Dakewe), respectively.

The secretions of IFN-γ after mixed culture of DC cells and T cells were shown in Figures 50-Figure 53. The secretions of IL-2 after mixed culture of DC cells and T cells were shown in Figures 54-56.

As shown in figures, the antibodies 4G10H1L1, 4G10H3L3, and 14C12H1L1, as well as bispecific antibodies BiAb001, BiAb002, BiAb003, and BiAb004 all can effectively induce the secretion of IFN-γ and IL-2 in mixed lymphocytes. The IFN-γ secretion induced by 1 nM or 10 nM anti-PD-1 antibody 14C12H1L1 were comparable with that of 100 nM control antibody Nivolumab. The IFN-γ secretion induced by 100 nM anti-CTLA4 antibodies 4G10H1L1 and 4G10H3L3 were better than that of 100 nM control antibody Ipilimumab (Figure 52).

Example 12: Induced IL-2 Secretion

The isolated PBMCs (the same method as in Example) was stimulated with PHA (Shanghai Shenqi Biotech Co., Ltd, 50 μl/ml) for 3 days, and then PBMCs (from volunteer blood donors, 5×10^4 cells/well) mixed with Raji cells (from ATCC, 5×10^4 cells/well) and MDA-MB-231 cells (from ATCC, 1×10^4 cells/well) in a 96-well plate. Antibodies (100 nM) were added and mixed and cultured together. After co-culture for 3 days, secretion of IL-2 was measured with ELISA reagent kit (purchased from Dakewe) according to the instructions.

The IL-2 secretion after mixed cell culture was shown in Figures57, Figure 58, and Figure- 59, respectively. As shown in the figures, the antibodies 4G10H1L1, 4G10H3L3, and 14C12H1L1, as well as bispecific antibodies BiAb001, BiAb002, BiAb003, and

BiAb004 can effectively induce the secretion of IL-2 by PBMCs. The anti-PD-1 antibody 14C12H1L1 can induce a higher IL-2 secretion than the control antibody Nivolumab (Figure 58), and bispecific antibodies BiAb001, BiAb002, BiAb003, and BiAb004 have the same effects on IL-2 secretion as 14C12H1L1+4G10H1L1 or 14C12H1L1+4G10H3L3 (Figure 59).

Example13: Impact of Antibody BiAb004 on the Tumor Growth of MC38 Tumor Model in PD-1 HuGEMM Mice

MC38 tumor cells were inoculated subcutaneously on the right side of PD-1 HuGEMM mice (1×10^6 cells/mouse, human PD-1 transgenic mice). When the mean tumor volume reached approximately 144 mm^3, the mice were randomly divided into 4 experimental groups per tumor volume with 8 mice in each group. Antibodies were given through abdominal administration, the specific grouping and dosages were as follows:

Isotype Control group (dose: 2.67 mg/kg),

BiAb004 high-dose group (dose: 2.67 mg/kg),

BiAb004 low-dose group (dose: 0.267 mg/kg),

The above 3 groups were injected with antibodies twice weekly, 5 times in total. After injection, the tumor sizes were measured twice weekly.

The results were presented in Figure 60.

Evidently:

The tumor sizes in the BiAb004 high-dose, and BiAb004 low-dose groups were all significantly smaller than those in the Isotype control group statistically ($P < 0.001$, $P < 0.05$, respectively). BiAb004 low-dose groups showed a statistically significant antitumor effect on the MC38 tumor model in the PD-1 HuGEMM mice.

Although specific embodiments of the present invention have been described in detail, as will be appreciated by one skilled in the art, these details may incur various modifications and substitutions according to all the teachings we have disclosed. These changes are all covered by the scope of the present invention. The full scope of the present invention is given by the appended claims and any equivalents.

Claims

1. A bispecific antibody comprising at least one first protein functional area that binds to PD-1 and at least one second protein functional area that binds to CTLA4, wherein the at least one first and at least one second protein functional areas each comprise an immunoglobulin or antigen-binding fragment thereof,

wherein the at least one first protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 29-31 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 32-34,

wherein the at least one second protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35-37 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40.

2. A bispecific antibody comprising at least one first protein functional area that binds to PD-1 and at least one second protein functional area that binds to CTLA4, wherein the at least one first and at least one second protein functional areas each comprise an immunoglobulin or antigen-binding fragment thereof,

wherein the at least one first protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 29-31 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 32-34,

wherein the at least one second protein functional area comprises comprising a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35, 41 and 37 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40.

3. A bispecific antibody comprising at least one first protein functional area that binds to PD-1 and at least one second protein functional area that binds to CTLA4, wherein the

at least one first and at least one second protein functional areas each comprise an immunoglobulin or antigen-binding fragment thereof,

wherein the at least one first protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 29-31 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 32-34,

wherein the at least one second protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 42-44 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 45-47.

4. The bispecific antibody of any one of claims 1-3, wherein the heavy chain variable region of the at least one first protein functional area comprises the amino acid sequence of SEQ ID NO: 16 or SEQ ID NO: 20 and the light chain variable region of the at least one first protein functional area comprises the amino acid sequence of SEQ ID NO: 18 or SEQ ID NO: 22.

5. The bispecific antibody of claim 1, wherein the heavy chain variable region of the at least one second protein functional area comprises the amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 6 or SEQ ID NO: 10 and light chain variable region of the at least one second protein functional area comprises the amino acid sequence of SEQ ID NO: 4, SEQ ID NO: 8 or SEQ ID NO: 12.

6. The bispecific antibody of claim 2, wherein the heavy chain variable region of the at least one second protein functional area comprises the amino acid sequence of SEQ ID NO: 14 and light chain variable region of the at least one second protein functional area comprises the amino acid sequence of SEQ ID NO: 12.

7. The bispecific antibody of claim 3, wherein the heavy chain variable region of the at least one second protein functional area comprises the amino acid sequence of SEQ ID

NO: 25 and light chain variable region of the at least one second protein functional area comprises the amino acid sequence of SEQ ID NO: 27.

8. The bispecific antibody of any one of claims 1-7, wherein,
the antigen-binding fragment comprises a half antibody, a Fab, a F(ab')2 or a single-chain antibody.

9. The bispecific antibody of any one of claims 1-8, wherein the at least one first protein functional area comprises an immunoglobulin and the at least one second protein functional area comprises a single-chain antibody.

10. The bispecific antibody of any one of claims 1-8, wherein the at least one first protein functional area comprises a single-chain antibody and the at least one second protein functional area comprises an immunoglobulin.

11. The bispecific antibody of any one of claims 1-10, wherein the bispecific antibody comprises at least two or more first protein functional areas, at least two or more second protein functional areas or any combination thereof.

12. The bispecific antibody of any one of claims 1-11, wherein the immunoglobulin is IgG, IgA, IgD, IgE or IgM.

13. The bispecific antibody of claim 12, wherein the immunoglobulin is IgG.

14. The bispecific antibody of claim 13, wherein the immunoglobulin is IgG1, IgG2, IgG3 or IgG4.

15. The bispecific antibody of any one of claims 9-14, wherein the single-chain antibody is attached at the c-terminus of the heavy chain of the immunoglobulin.

16. The bispecific antibody of any one of claims 1-15, wherein the immunoglobulin or antigen-binding fragment thereof contains non-CDR regions from species other than mouse, and optionally, from human.

17. The bispecific antibody of any one of claims 1-16, wherein the bispecific antibody binds to CTLA4 protein and/or PD-1 protein with a K_D less than approximately 10^{-5} M, and optionally, less than approximately 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M, 10^{-10} M or less.

18. A composition comprising isolated nucleic acid molecules encoding for the bispecific antibody of claim 1, wherein the composition comprises

a first isolated nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain variable region of an antibody and a single-chain antibody, wherein

the heavy chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 29-31,

and wherein the single-chain antibody comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35-37 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40; and

a second isolated nucleic acid molecule comprising a nucleotide sequence encoding the light chain variable region of an antibody, wherein,

the light chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 32-34.

19. A composition comprising isolated nucleic acid molecules encoding for the bispecific antibody of claim 2, wherein the composition comprises

a first isolated nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain variable region of an antibody and a single-chain antibody, wherein

the heavy chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 29-31,

and wherein the single-chain antibody comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35, 41 and 37 and a

light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40; and

a second isolated nucleic acid molecule comprising a nucleotide sequence encoding the light chain variable region of an antibody, wherein,

the light chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 32-34.

20. A composition comprising isolated nucleic acid molecules encoding for the bispecific antibody of claim 3, wherein the composition comprises

a first isolated nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain variable region of an antibody and a single-chain antibody, wherein

the heavy chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 29-31,

and wherein the single-chain antibody comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 42-44 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 45-47; and

a second isolated nucleic acid molecule comprising a nucleotide sequence encoding the light chain variable region of an antibody, wherein,

the light chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 32-34.

21. The composition of any one of claims 18-20, wherein the first isolated nucleic molecule and the second isolated nucleic molecule are present in a single vector.

22. The composition of any one of claims 18-20, wherein the first isolated nucleic molecule is present in a first vector and the second isolated nucleic molecule are present in a second vector.

23. A host cell line, comprising the composition of any one of claims 18-22.

24. A method for preparing the bispecific antibody of any one of claims 1-17, the method comprising culturing the host cell line of claim 23 under appropriate conditions, and recovering the bispecific antibody from the cell culture.

25. A conjugate comprising the bispecific antibody of any one of claims 1-17 and a conjugating partner as a detectable marker.

26. The conjugate of claim 25, wherein the conjugating partner is a radioactive isotope, fluorescein, a luminescent material, a colorful substance, or an enzyme.

27. A kit comprising the bispecific antibody of any one of claims 1-17 or the conjugate of any one of claims 25-26.

28. The kit of claim 27, wherein the kit further comprises a secondary antibody, wherein the secondary antibody specifically recognizes the bispecific antibody.

29. The kit of claim 28, wherein the secondary antibody comprises a detectable marker.

30. The kit of claim 29, wherein the detectable marker is a radioactive isotope, fluorescein, a luminescent material, a colorful substance, or an enzyme.

31. A pharmaceutical composition, comprising the bispecific antibody of any one of claims 1-17 or the conjugate of any one of claims 25-26.

32. The pharmaceutical composition of claim 31, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable carrier or excipient.

33. Use of the bispecific antibody of any one of claims 1 -17, the conjugate of any one of claims 25-26 or the pharmaceutical composition of any one of claims 31-32 for the manufacture of a medicament for the treatment of a tumor.

34. The use of claim 33, wherein the tumor is selected from the group consisting of melanoma, renal cancer, prostate cancer, bladder cancer, colorectal cancer, gastrointestinal cancer, liver cancer, non-small cell lung cancer, ovarian cancer and leukemia.

35. Use of the bispecific antibody of any one of claims 1-17, the conjugate of any one of claims 25-26 or the pharmaceutical composition of any one of claims 31-32 for the manufacture of a medicament for the treatment of a viral or parasitic infection.

36. The use of any one of claims 33-35, wherein the bispecific antibody or the conjugate is to be administered to the subject in at least one therapeutically effective amount.